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Date

Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Roth et al.

Serial No.: 08/918,407

Filed: August 26, 1997

For: METHODS AND COMPOSITIONS COMPRISING DNA DAMAGING AGENTS AND p53

Group Art Unit: 1636

Examiner: Sandals, William O.

Atty. Dkt. No.: INRP:050

DECLARATION OF DEBORAH R. WILSON, PH.D, UNDER 37 C.F.R. 1.132

I, Deborah R. Wilson, Ph.D, declare that:

1. I am the Associate Vice President of Clinical Research at Introgen Therapeutics, Inc. ("Introgen"), assignee of the above-captioned application. I have been employed at Introgen for 7 years and was recently named Associate Vice President. My responsibilities as Associate Vice President of Clinical Research at Introgen include clinical science, pharmacokinetics, and drug safety. I am a citizen of the United States of America, and I reside at 11022 Silkwood, Houston, Texas 77031.

2. I understand that the Patent and Trademark Office has rejected claims in the above-referenced case as lacking enablement, based on reasons related to the lack of success of gene therapy.

3. Introgen and its collaborators have been conducting research and development of an Ad-p53 composition for the treatment of cancer for almost a decade. Introgen's research and development has progressed to the point where its Ad-p53 composition, INGN 201 (Introgen's Advexin® adenovirus p53 product), which is disclosed in the present application, is involved in a number of clinical trials for head and neck cancer, lung cancer, breast cancer, esophageal cancer, glioma, prostate cancer, advanced solid tumors, bladder cancer, and ovarian cancer. *See* Table of Adenovirus-p53 Clinical Trials (Exhibit 1). INGN 201 is in phase III clinical trials for head and neck cancer. Phase II clinical trials are underway or have been completed for head and neck cancer, esophageal cancer, breast cancer, and non-small cell lung carcinoma. INGN 201 was used or has been approved for phase I clinical trials for lung cancer, breast cancer, liver cancer, glioma, prostate cancer, head and neck cancer, bladder cancer, ovarian cancer, colorectal cancer, malignant ascites, and solid tumors from a variety of origins.

4. Several clinical trials have been conducted for various cancers including ovarian cancer, lung cancer, bladder cancer, and metastatic colorectal cancer using a different Ad-p53 construct from another company, Schering Plough.[1]

5. The clinical trials discussed in paragraphs 3 and 4 involved or will involve a variety of administrations of Ad-p53 constructs. Administrations include: intraperitoneal, intravenous, intravesical, intratumoral, intramucosal injection, oral rinse, and broncho-alveolar lavage.

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[1] *See. e.g.*, Barnard (2000); Horowitz (1999); Kuball *et al.* (2002); Schuler *et al.* (2001) and the reference of Wills *et al.*, which provides the details regarding the structure of the SCH 58500 Ad-p53 construct, which lacks protein IX. (Exhibit 2)

6. I anticipate Introgen will proceed with other clinical trials in the future involving adenovirus-p53 constructs, given the success I have observed in the ongoing or previous clinical trials with Introgen's product, INGN 201.

7. I declare that all statements made herein of my own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this patent, and any reexamination certificate issuing thereon.

15 April 2002
Date

Deborah R. Wilson, Ph.D.

25156121.1

#39 attachments

Table of Adenovirus-p53 Clinical Trials (as of February 2002)

Treatment	Cancer	Admin	Clinical Stage	Status/Result
INGN 201	SCCHN (T302)	Intratumoral	III	Ongoing
INGN 201	SCCHN (T301)	Intratumoral (with chemotherapy)	III	Ongoing
INGN 201	NSCLC	Intratumoral (with radiation therapy)	II	Combination INGN 201 and radiation therapy appears more effective than radiation alone
INGN 201	SCCHN (T207)	Intratumoral	II	Safe
INGN201	Locally advanced primary breast	Intratumoral (with chemotherapy)	II	Study has been initiated
INGN 201	Esophageal	Intratumoral	II	Ongoing
INGN 201	SCCHN (T201)	Intratumoral	II	Safe; demonstrated clinical activity
INGN 201	SCCHN (T202)	Intratumoral (lower dose)	II	Safe; trend towards shorter survival than T201
INGN 201	Ovarian	Intraperitoneal	I	Transgene expression observed and increased expression of downstream marker; well-tolerated
INGN 201	Ovarian	Intraperitoneal (laparoscopy)	I	Well-tolerated; potentially useful clinical response
INGN 201	Bladder	Intravesical	I	Transgene expression observed; safe; ongoing
INGN 201	Advanced solid tumors (colon, breast, prostate, sarcoma,	Intravenous	I	Well tolerated at doses up to $1x10^{12}$ vp; accrual is ongoing to further

	NSCLC, H&N)			determine MTD; evaluation of p53 expression is pending
INGN 201	SCCHN	Intratumoral (with and without tumor resection)	I	Transgene expression and expression of downstream targets observed; safe; potentially useful clinical response
INGN 201	NSCLC	Intratumoral	I	Transgene expression and apoptosis observed; safe; potentially useful clinical response
INGN 201	NSCLC	Intratumoral (with cisplatin)	I	Expression observed; well tolerated; potentially useful clinical response
INGN 201	Prostate	Intratumoral (INGN 201 treatment prior to tumor resection)	I	Transgene expression and apoptosis demonstrated; safe
INGN 201	Glioma	Intratumoral and intracranial (stereotactic injection intratumorally, followed by tumor resection, followed by injection into tumor bed	I	Expression observed; safe; apoptosis observed; ongoing
INGN 201	Hepatocellular Carcinoma	Intratumoral	I	Study closed; 1 patient treated
INGN 201	Breast	Intratumoral (with chemotherapy)	I	Study closed; 2 patients treated

INGN 201	Bronchioloalveolar lung carcinoma	Broncho-alveolar lavage	I	Safe; potentially useful clinical response; ongoing
INGN 201	Malignant ascites	Intraperitoneal	I	Study closed; 1 patient treated
INGN 201	Colorectal	Intratumoral	I	Study closed; 6 patients treated; expression of downstream markers demonstrated
INGN 201	Lung	Intratumoral (with and without cisplatin)	I	Ongoing
INGN 201	Oral dysplasia (premalignant)	Intramucosal injection; oral rinse		Not started
SCH 58500	Ovarian	Intraperitoneal (with chemotherapy)	II/III	Reported closed
SCH 58500	Lung	Intratumoral (with chemotherapy)	II	Transgene expression observed; well-tolerated; enhanced local effects suggested with certain chemotherapies
SCH 58500	Ovarian	Intraperitoneal (with chemotherapy)	I/II	Well tolerated; expression observed; prolonged patient survival
SCH 58500	Lung	Intratumoral	I	Transgene expression and expression of downstream target observed; safe; transient tumor growth control
SCH 58500	Bladder	Intratumoral or intravesical (with	I	Transgene expression and expression of downstream marker

		transduction enhancer)		demonstrated after intravesical instillation

Technology evaluation: Sch-58500, Canji

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Sch-58500 is a gene therapy utilizing the p53 gene and is under development by Canji and Schering-Plough for the potential treatment of various types of cancer. It is in phase II/III clinical trials in the US for stage III ovarian cancer [328228,328893], phase II clinical trials for hepatocellular and colorectal cancer metastatic to the liver [273331,324279], and phase I clinical trials for several other types of cancer [282801,284932,328228].

Originator Canji Inc

Licensees Genzyme Molecular Oncology, Schering-Plough Corp

Status Phase III Clinical

Indications Carcinoma, liver tumor, breast tumor, colorectal tumor, lung tumor, leukemia, head and neck tumor, melanoma, ovary tumor, neoplasm

Action Anticancer

Technology Gene therapy

Synonyms ACN-53; ACN-p53 TSG; Ad-p53; rAd/p53, Canji/Schering-Plough; gene therapy (p53), Canji/Schering-Plough

Introduction

Canji/Schering-Plough are currently evaluating a new antitumor gene therapy agent, Sch-58500. It is a recombinant adenovirus (Ad) expressing a human p53 apoptosis-inducing gene that triggers targeted tumor cells to undergo programmed cell death (apoptosis). Tumors that are especially sensitive to this type of therapy are those in which the normal cellular *p53* gene is not functional. For example, non-functional (mutant) p53 is expressed in 50% of patients with primary breast tumors and their prognoses are significantly more grim than patients with breast tumors with functional *p53* genes [255115]. Approximately 50% or more of other types of tumors have a non-functional *p53* gene, which suggests that gene therapies such as Sch-58500 that could target p53 mutant cancer cells might have wide utility in the treatment of cancers. The virus has been designed to infect susceptible cells, which include many types of tumor cells [246633,359068]. By doing so, the virus causes the infected cell to transcribe the *p53* gene and translate the message to a functional protein product that will cause the tumor cell to undergo apoptosis [246632,284805]. Because many cancer cell lines were inhibited in cell culture, Sch-58500 is now being tested in clinical trials in patients with a variety of tumors.

Vector

The vector of the *p53* gene, Ad type 5 (Ad5) is a replication-deficient virus, which lacks the genes needed for infectious virus replication, ie, *p9*, *E1*, *E1A* and *E3* [168287]. As a result, the virus can infect a cell but cannot direct that cell to replicate infectious virus progeny. However, the virus can deliver a functional *p53* gene to any cell to which it can attach and penetrate. Once uncoated, the *p53* gene can be transcribed and translated into a functional gene product that will trigger an apoptotic pathway. The *p53* gene was inserted into the Ad genome, along with a cytomegalovirus promoter that facilitates efficient transcription of the *p53* gene [284805].

Pharmacology

The antitumor activity of Sch-58500 probably results from several modes of inhibition. Firstly, the vector delivers the *p53* gene to any cell that it can infect. The subsequent transcription and translation of the *p53* gene results in an apoptosis-inducing protein product leading to programmed cell death [246630,284932]. Secondly, specialized immune cells, called natural killer cells, are activated at the site of the tumor, which destroy the tumor cells infected with virus vector and some 'bystander cells' at the site of virus infection [380033]. Thirdly, the vector itself mediates tumor growth suppression [168287]. In addition, animal studies have demonstrated that vector also reduces the spread or metastatic potential of the tumor cells that become infected with the vector [255115,284805]. In addition, Sch-58500 increases the sensitivity of tumor cells to inhibition by chemotherapeutic agents such as 5-fluorouracil, cisplatin, etoposide, doxorubicin and paclitaxel (National Institutes of Health) [380035]. In tumor-bearing animals, paclitaxel acted synergistically with Sch-58500 to kill tumors. The mode of action of this synergism appeared to be an enhancement of the uptake of Sch-58500 into the tumor cells, thereby increasing the effective p53 concentration [284304].

The vector is usually administered in the dose range of 1×10^9 to 7.5×10^{12} total virus particles, depending on the type of tumor [308167]. The mode of delivery is also tumor-dependent, eg, it can be directly injected into brain, head or neck tumors, or intraperitoneally for ovarian cancers, or via the hepatic artery for liver cancers [339443,380035,380041]. The vehicle for delivery is often normal saline. When injected, the vector can penetrate one to ten cell layers deep, depending on the nature of the tissue [380042].

Sch-58500 reduced the spread of certain tumors in immunocompromised mice by 60 to 80% [255115]. The importance of tumor growth suppression of the Ad vector itself was demonstrated in mice that were treated with the potent immunosuppressant, dexamethasone. Tumor growth was still suppressed by the p53-mediated mechanism, but not by the virus-mediated suppression of growth [380033]. Suppression of NK cell response with antibody that neutralized NK cells gave the same result, ie, inhibition of

NK-mediated cell destruction but no effect on p53-induced programmed tumor cell [380033].

Combination therapy with other antitumor drugs has also been evaluated in cell culture. Sch-58500 was used in combination with Sch-66336 (Schering-Plough Research Institute). The latter compound is a farnesyl transferase inhibitor that inhibits the addition of a farnesyl group on RAS proteins, which is an intermediate in an apoptosis pathway [325867,359068]. This combination was a more potent antitumor therapy than either drug alone. Greater efficacy of inhibition was achieved against DU-145 human prostate and *wap-ras/F* transgenic mouse cancer models for Sch-58550 in combination with other agents [380033]. The combination of Sch-58500, cisplatin and paclitaxel was particularly potent in an ovarian cancer animal model. Additionally, Sch-58500 in combination with FDA approved drugs such as cisplatin, doxorubicin, 5-fluorouracil, methotrexate and etoposide resulted in a more potent suppression of tumor cell proliferation in SSC-9, SSC-15 and SSC-25 head and neck tumor cells, SK-OV-3 ovarian tumor cells, DU-145 prostate tumor cells, MDA-MB-468 and MDA-MB-231 breast cancer cells [168287]. Greater anticancer activity was also observed when four human tumor xenografts growing in mice were treated with these combinations [380035]. Sch-58500 was also shown to be safe and efficacious in preventing tumor growth of a human colorectal adenocarcinoma in *nu/nu* immunodeficient mice without thymuses (reduced cellular immunity) [246635]. The mechanisms whereby a synergistic or additive effect was achieved may be due to one drug enhancing the uptake of another, or one drug enhancing the sensitivity of the tumor cells to inhibition by another drug. For instance, it was found that the paclitaxel enhanced the uptake of Sch-58500 [284304], and that Sch-58500 rendered certain tumor cells susceptible to the other drugs used in the combination therapy [284304].

Toxicity

Sch-58500 was generally well tolerated. However, there are concerns about the adverse effect of similar Ad vectors, arising from the death of a patient receiving gene therapy for ornithine transcarbamylase deficiency (OTCD). This has led the FDA to request discontinuation of patient enrollment in several clinical studies of Sch-58500 as a precautionary measure until the reason for patient death is determined [343174,343175].

Clinical Development

Phase I

A phase I/II clinical trial was carried out to evaluate the efficacy of Sch-58500 in recurrent ovarian cancer [380044], but this has since been discontinued. Another phase I study was used to determine the safety and efficacy of Sch-58500 in patients with advanced head and neck cancer [339443]. In this study, Sch-58500 was administered by single or multiple injection to patients with tumors amenable to injection therapy. They received four dose levels of virus based on total virus particles injected. In preliminary results, functional p53 DNA was detected in tumors by reverse transcriptase-polymerase chain reaction (RT-PCR) in four of ten patients whose results were available [339443].

A phase I trial was carried out in 64 patients with hepatic metastases of colon cancer, head and neck cancer, ovarian cancer and melanoma [284304]. Transgenic expression of p53 was detected by RT-PCR and was dose-dependent. Antibody against the Ad vector was also detected, although it did not influence the expression of p53 within the tumor. In another phase I trial involving 62 patients (including those with the tumors described above, as well as patients with non-small-cell lung carcinoma), 30 of 57 patients expressed normal p53 at the tumor site [282801].

70 Patients with brain cancer, head and neck cancer and ovarian cancer were enrolled in an efficacy trial [284932]. Those with the head/neck and brain cancers received 10^9 to 10^{12} virus particles in one administration. Biopsies were taken from 69 of the patients, and in 33 the p53 transcript was detected in a dose-responsive manner. The more particles that were given, the larger amounts of p53 were detected.

In a trial to determine toxicity, gene expression and immune response in patients with primary metastatic liver cancer or ovarian cancer, Sch-58500 was administered by hepatic artery injection or intraperitoneally, respectively [306641]. This was a single/multidose trial either with Sch-58500 alone or in combination with traditional anticancer agents. Antibody to the virus vector was detected but did not affect the dose-dependent expression of p53 at the tumor site. Some mild to moderate side effects were observed, which included fever, malaise and anemia. In another study, 30 patients with hepatocellular carcinoma received a single dose of 7.5×10^9 to 7.5×10^{13} virus particles [312158]. Here, moderate toxicity was noted, which included the standard symptoms described previously, along with tachycardia and hypertension. Greater expression of p53 was detected at the tumor site than in the normal cells, although the apoptotic index was the same on both the treated side of the liver containing the tumor cells and the non-treated side of the liver. This indicated that normal cells were not adversely affected by the treatment and that tumor cells not previously expressing p53 did express p53.

A combination, single/multiple dose study was undertaken in 41 ovarian cancer patients [312158]. Patients receiving one dose were injected with 10^{10} or 10^{12} particles, and those receiving multiple doses received 10^{12} to 10^{13} virus particles. The recipients of the combination therapy were injected with platinum ip, or with taxol/carboplatin iv. There were moderate side effects, including nausea and vomiting, and p53 was detected at the tumor site. 80 Patients with head and neck tumors received single and multiple doses of Sch-58500 (10^9 to 10^{13} virus particles) and combination therapy via several routes of inoculation [328228]. The maximum tolerated dose in these patients was dependent on the route of administration, with ip administration giving best results. When administered iv, some lymphopenia and thrombocytopenia were detected. Vector shedding also occurred.

Another phase I ovarian cancer study using single and multiple dosing regimens and combination therapy was initiated in 36 patients [347524]. For this study, drugs were administered ip; although moderate toxicity was noted

(nausea, fatigue, hypotension, fever and anemia), there was a reduction in CA-125 tumor marker in 54% of the patients.

Phase II/III

A number of phase II and phase II/III clinical trials have been planned or have commenced. A phase II study in patients with primary or metastatic liver tumors begun in 1998 [328228]. These patients received multiple high doses of Sch-58500 alone or in combination with traditional antitumor agents, either via the hepatic artery or ip; this study has now been discontinued

A phase II study was carried out to determine the efficacy of Sch-58500 in combination with traditional chemotherapeutic drugs in patients with liver and colorectal cancers. The patients were implanted with a pump to deliver the standard drug therapies, such as FLDTR, dexamethasone and Leucovir. In 1999, the FDA asked Canji and Schering-Plough to cease this trial temporarily because of safety concerns that arose from an unrelated trial in which another company was testing an Ad vector and death of a patient had occurred [343174,343175].

A multicenter phase II trial of Sch-58500 in patients with colorectal cancer metastatic to liver commenced in May 1999, along with a trial in patients with hepatocellular cancer metastatic to liver [324279]. Several phase II/III studies were started in 1999 and 2000, including study of the effect of common chemotherapeutic agents used alone or in combination with Sch-58500 ip in newly diagnosed ovarian cancers with the *p53* mutation [328228,328893].

Side Effects and Contraindications

Most trials report few side effects with Ad-p53 when used alone, relative to the adverse effects of chemotherapy. However, in a report a pancreatic cancer trial in Germany, where the vector was introduced intra-arterially, several patients experienced mild disseminated intravascular coagulation [368768]. Additionally, there is concern about safety following the death of a patient from the University of Pennsylvania study of Ad vector containing a gene intended to correct OTCD. Since Sch-58500 conceptually uses the same type of vector, the FDA has asked Schering-Plough to discontinue enrolling patients in several studies until the reason for this death is determined.

Current Opinion

The use of Ad gene delivery vector represents an exciting approach to eliminating a variety of tumors deficient in the *p53* apoptosis-inducing gene. The Canji/Schering-Plough Ad vector appears to be relatively free of side effects when administered alone, and in combination with existing chemotherapeutic agents it offers an enhanced, less toxic alternative to traditional chemotherapeutics. This evaluation, however, concludes with a note of caution Because of the death of a patient who was administered a related Ad vector in an trial conducted at the University of Pennsylvania, further new trials of Ad-based therapeutic agents have been temporarily suspended at the request of the FDA [343174]. These trials remain on hold, pending demonstration that an appropriate oversight and clinical monitoring program is in place [380063].

Licensing

Genzyme Molecular Oncology

In January 1998, Schering-Plough entered into a research collaboration with Genzyme Molecular Oncology (GMO) to develop gene therapies using GMO's lipid delivery system. The first year of the collaboration focused on the development of a delivery system for the gene p53. Schering-Plough will subsequently have the option of exclusive license to the technology for other, as yet undisclosed, gene therapies [273382].

Schering-Plough Corp

In October 1994, Schering-Plough formed an alliance with Canji to develop new cancer treatments based on Canji's p53 gene therapy technology. The agreement grants affiliated companies of Schering-Plough exclusive worldwide licenses to make, use and sell p53 tumor-suppressor gene products for human and animal uses. Under the agreement, Schering-Plough made an initial cash investment in Canji and was to make annual, performance and milestone payments over the next several years [168987]. Canji was acquired by Schering-Plough in 1996 [197761].

Development History

DEVELOPER	COUNTRY	STATUS	INDICATION	DATE	REFERENECE
Canji Inc	US	C3	Ovary tumor	23-JUN-99	323393
Canji Inc	US	C2	Lung tumor	05-JAN-98	273331
Canji Inc	US	C2	Colorectal tumor	12-MAY-99	324279
Schering-Plough Corp	US	C2	Lung tumor	01-JAN-98	273331
Schering-Plough Corp	US	C2	Colorectal tumor	12-MAY-99	324279
Canji Inc	US	C1	Liver tumor	01-JAN-96	237334
Canji Inc	US	C1	Melanoma	01-JAN-96	237334

Development History (continued)

DEVELOPER	COUNTRY	STATUS	INDICATION	DATE	REFERENCE
Canji Inc	US	C1	Neoplasm	01-JAN-96	237334
Canji Inc	US	C1	Breast tumor	01-JAN-96	237334
Canji Inc	US	C1	Head and neck tumor	01-JAN-96	237334
Schering-Plough Corp	US	C1	Melanoma	01-JAN-96	237334
Schering-Plough Corp	US	C1	Breast tumor	01-JAN-96	237334
Schering-Plough Corp	US	C1	Head and neck tumor	01-JAN-96	237334
Schering-Plough AB	US	C1	Liver tumor	01-JAN-96	237334
Schering-Plough AB	US	C1	Ovary tumor	01-DEC-98	315026
Schering-Plough Corp	US	DR	Leukemia	01-JAN-95	182509
Genzyme Molecular Oncology	US	DR	Neoplasm	17-MAR-98	279382
Canji Inc	US	DR	Leukemia	01-JAN-95	182509

Literature Classifications

Key references relating to the technology and are classified according to a set of standard headings to provide a quick guide to the bibliography. These headings are as follows:

Chemistry. References which discuss synthesis, construction and structure-activity relationships.

Biology: References which disclose aspects of the drugs pharmacology in animals

Metabolism: References which discuss metabolism, pharmacokinetics and toxicity.

Clinical: Reports of clinical phase studies in volunteers providing, where available, data on the following: whether the experiment is placebo-controlled or double- or single blind; number of patients; dosage.

Chemistry

STUDY TYPE	RESULTS	REFERENCE
Construction.	Successful construction of the rAd/p53 vector expressing p53 in a dose-dependent manner in cancer cells in culture.	168287
Purification.	Column chromatography purification of rAd/p53 vector expressing p53.	246629

Biology

STUDY TYPE	EFFECT STUDIED	EXPERIMENTAL MODEL	RESULTS	REFERENCE
In vitro	Transduction of tumor cells.	Cell culture.	Successful transduction of tumor cells with rAd/p53.	246630
In vivo	Expression.	Nude mice.	Successful *ex vivo* treatment of Saos-2 tumor cells followed by injection into nude mice resulted in complete tumor suppression using the rAd/p53 vector.	168287
In vivo	Expression.	Mouse xenografts.	Induction of apoptosis and growth reduction using the rAd/p53 vector to treat against 231 and 468 tumor xenografts.	246630
In vivo	Antitumor effect.	SCID-beige mice.	Reduction of the metastases of lung. Reduction of tumors in mice.	255115
In vivo	Penetrability.	Mouse tumor xenografts.	Depth of penetration of Sch-58500 determined in tumor tissue.	380042
In vivo	Dosage.	Human tumors.	Effective dosage range determined.	308167

Biology (continued)

STUDY TYPE	EFFECT STUDIED	EXPERIMENTAL MODEL	RESULTS	REFERENCE
In vitro	Synergy.	Cultured tumor cells.	Enhanced sensitivity of tumor cells to anticancer agents when treated in combination with Sch-58500.	380035
In vivo	Tissue penetration/ permeability.	Human tumors.	Enhanced permeability of tumors to Sch-58500 when used in combination with paclitaxel.	284304

Metabolism

STUDY TYPE	EFFECT STUDIED	EXPERIMENTAL MODEL	RESULTS	REFERENCE
In vivo	Tissue distribution.	Human tumor biopsies.	Expression of p53 in p53-negative tumors from biopsies of patients treated with rAd/p53.	284932
In vivo	Tissue distribution.	Human tumor biopsies.	Immunohistochemical evidence of p53 expression in tumors and efficacy, despite antibody formation to the Ad5 vector.	339443

Clinical

EFFECT STUDIED	EXPERIMENTAL MODEL	RESULTS	REFERENCE
Phase III. Safety/biological activity.	Head and neck cancer trial ongoing, but no grade 3/4 toxicities reported.	Functional p53 DNA was detected in tumors by RT-PCR in four of ten patients whose results were available.	339443
Phase I/II. Safety/biological activity.	Variety of human tumors.	Transgene expression was detected by RT-PCR in many tumors of patients despite an antibody response to the adenovirus vector.	284304

Associated Patent

Title Gene therapy using replication competent targeted adenoviral vectors.

Assignee Canji Inc

Priority US-08433798 3-MAY-95

Publication WO-09634969 7-NOV-96

Inventors Gregory RJ, Huang W-M

Abstract

A novel method of treating cancer is claimed. The method involves the use of a replication-competent targeted Ad vector. The vector preferentially replicates in tumor cells due to activation of a tumor-specific gene regulatory region. These vectors can be used as a form of gene therapy to deliver therapeutic genes to treat cancer. Ad vectors were constructed using standard techniques to place the *E1a* gene under the control of a tumor-specific promoter, AFP. Therapeutic genes, such as a cytotoxic gene, were inserted into the Ad E3 region. The Ad vectors were assessed for their replication potential in tumor cell lines that can and cannot utilize the AFP promoter. The vectors of this invention were at a replication disadvantage compared to wild-type Ad in the AFP-negative cell line, HLE. However, they were able to replicate more efficiently in AFP-positive tumor cell lines.

References

• of special interest

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• *Describes the construction of the adenovirus vector with the p53 gene inserted, which is known as Sch-58500.*

168987. Schering-Plough Corp: **Schering Plough, Canji announce agreement to develop gene therapy cancer treatments.** *Press release* 26 October (1994).

197761. Schering-Plough Corp: **Schering-Plough completes acquisition of Canji Inc.** *Press release* 2 February (1996).

246630. Nielsen LL, Dell J, Maxwell E, Armstrong L, Maneval D, Catino JJ. **Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts.** *Cancer Gene Ther* (1997) 4(2):129-138.
• *A study demonstrating that one mode of tumor inhibition by Sch-58500 is the induction of programmed cell death in susceptible tumor cells.*

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• *Explains the mechanism of tumor inhibition by Sch-58500.*

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• *A study demonstrating the safety and efficacy of Sch-58500 in immunodeficient mice.*

255115. Gumani M, Dell J, Nielsen LL. **Efficacy of Sch-58500 in a metastatic model of human breast cancer.** *Proc Annu Meet Am Assoc Cancer Res* (1997) 38:Abs 84.
• *A study showing that reintroduction of a wild-type p53 gene into tumors not expressing p53 is feasible.*

273382. Schering-Plough Corp: **Schering-Plough and Genzyme Molecular Oncology collaborate on gene therapy delivery technology.** *Press release* 5 January (1998).

282801. Schering-Plough Corp: **Schering-Plough presents findings of p53 gene therapy studies at American Association for Cancer Research Annual Meeting.** *Press release* 1 April (1998).
• *A phase I clinical study demonstrating the expression of p53 in a variety of tumors.*

284804. Banerjee D. **American Association For Cancer Research 89th Annual Meeting (Part I): Cancer Gene Therapy, New Orleans, LA, USA.** *IDdb meeting report* (1998).
• *A study describing the mode of synergistic tumor inhibition by Sch-58500 and chemotherapeutic anticancer agents.*

284805. Horowitz J, Fritz MA, Swanson S, Petrauskas S, Borgens R, Rybak ME: **Transgene expression from the phase I pilot program rAd/p53 (Sch-58500).** *Proc Annu Meet Am Assoc Cancer Res* (1998) 39:Abs 361.
• *Describes the main mechanism of tumor inhibition by Sch-58500, ie, induction of apoptosis.*

284932. Hutchinson E: **American Association For Cancer Research 89th Annual Meeting (Part III); Cell-based gene therapy and late-breaking news, New Orleans, LA, USA.** *IDdb meeting report* (1998).
• *A phase I clinical study demonstrating that one mode of tumor inhibition by Sch-58500 is the induction of programmed cell death in susceptible tumor cells.*

306641. Schering-Plough Corp. **Schering-Plough reports findings of p53 gene therapy studies at conference on gene therapy of cancer annual meeting.** *Press release* 23 November (1998).
• *A study measuring toxicity, gene expression and the immune response in patients with primary metastatic liver cancer or ovarian cancer.*

308167. Fong TC: **Gene Therapy of Cancer - Seventh International Conference, Coronado Island, CA, USA.** *IDdb meeting report* (1998).
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Adenovirus-mediated p53 gene therapy: Overview of preclinical studies and potential clinical applications

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Disruption of p53 function through mutation, or other means, occurs very frequently in human cancer and is associated with an unfavorable prognosis in various cancers. Evidence from in vitro and in vivo transduction experiments have demonstrated that adenoviral-mediated expression of wild-type p53 suppresses the transformed phenotype of many cell types and potentiates the cytotoxicity of both chemotherapeutic agents and radiation therapy. Recently several phase I studies have evaluated the safety, biological effect and different routes of administration of adenoviral-mediated p53 gene therapy in various tumor types. These studies indicate that adenovirus-mediated p53 gene therapy and introduction of wild-type p53 into tumor cells represents a potentially valuable tool for the therapy of many types of human cancers. This review presents an overview of the most recent advances in the preclinical and clinical evaluation of adenoviral p53 gene therapy as well as the challenges that lay ahead for future clinical studies.

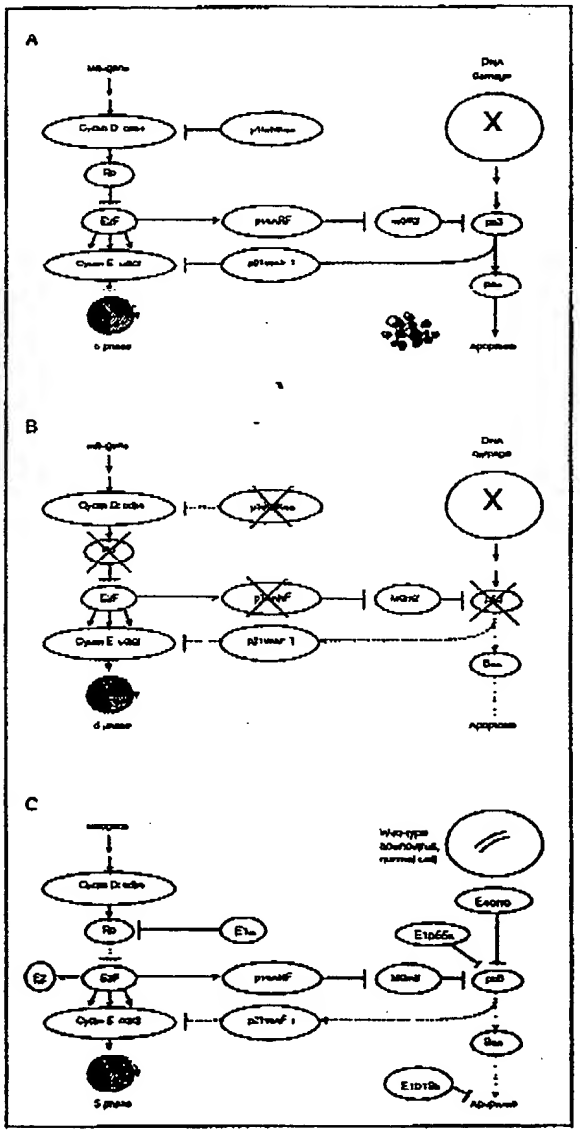
Introduction

Loss of p53 function appears to play a central role in a common pathway required for the development of most human cancers. p53 Mutations have been reported in nearly all tumor types and functional inactivation of p53 occurs in more than half of all cancers [1]. Although p53 is not required for normal development, patients with inherited p53 mutations (ie, Li-Fraumeni syndrome) and mice lacking one or both alleles of *p53* develop spontaneous tumors [2,3].

Inactivation of wild-type p53 can result from direct genetic mutations in the *p53* gene, binding of p53 protein by viral oncoproteins or cellular factors, or alteration of subcellular localization of p53 protein [4-8]. The inability of the cell to repair DNA damage leads to the accumulation of genetic changes that alter cellular responses to growth control. This has significant impact on the metastatic potential [9], as well as the response of tumor cells to therapeutic intervention [10,11]. In particular, the loss of p53 function has been associated with an unfavorable prognosis for cancers of the lung and breast, among others [12-14].

The *p53* tumor suppressor gene encodes a 393 amino acid nuclear phosphoprotein that plays a pivotal role in coordinating cellular responses to DNA damage and other forms of genotoxic stress. The p53 protein, a sequence-specific DNA transcription factor induces or represses the expression of multiple genes involved in regulating the cell cycle, DNA repair and apoptosis [6-8]. Activation of wild-type p53 in response to DNA damage either causes cell cycle arrest or induces apoptosis. While the cyclin-dependent kinase inhibitor p21WAF1/CIP1 mediates p53-induced cell cycle arrest, the induction of apoptosis can involve transcription-dependent (Bax, Fas) and/or independent signaling pathways (Figure 1) [15]. Although the exact signal

Figure 1. Signaling pathways activated by DNA damage to wild-type *p53*.



Signal pathways leading from p53 to cell growth arrest or apoptosis. (A) Signal pathway in normal cells. (B) Cancer cells have defects in the Rb and p53 pathway. (C) Wild-type adenovirus protein interference with the Rb and p53 pathways in infected normal cells. *(Cancer J Sci Am Vol 5, 1999 p139-144. Copyright 1999 American Cancer Society. Reprinted by permission of Wiley-Liss Inc, a subsidiary of John Wiley & Sons Inc.)*

pathways leading from p53 to cell growth arrest or apoptosis are not fully understood, they are clearly regulated in a tissue-specific manner [16].

The *p53* gene has become a target for the development of new therapeutic strategies for cancer. One approach currently under clinical investigation is the introduction of wild-type *p53* tumor suppressor gene into tumor cells to achieve tumor suppression. Preclinical studies have confirmed that the introduction of wild-type p53 into neoplastic cells results in growth suppression and reduction of colony formation in soft agar [17]. Studies in nude mice indicate that introduction of p53 into tumor cells reduces their tumorigenicity [17], induces apoptosis in tumor xenograft models [18], increases sensitivity to several chemotherapeutic agents [19] and inhibits angiogenesis [20].

Considerable effort has been expended to design an effective method for *p53* gene therapy using several different viral and non-viral gene delivery systems [21-23]. Selection of a delivery system for introduction of *p53* is important for efficient transduction and sufficient expression of functional p53 protein *in vivo*. The ideal vector for gene therapy would be available at high titers, be easily reproducible, and elicit little to no immune response. Early studies using retrovirus-mediated gene transfer of wild-type *p53* into both human lung cell lines and xenograft models demonstrated that expression of wild-type p53 could lead to inhibition of tumor cell growth. However, poor stability of these vectors and the inability to produce high titers of highly infective recombinant virus have limited the use of retroviruses as a gene delivery system for *p53* gene therapy [23]. More recent studies have focused on the use of adenoviruses and other non-viral gene delivery systems. The type 5 adenoviral vector is currently the vector of choice for *in vitro* and *in vivo* studies due to its ability to transduce both proliferating and quiescent cells, ease of manipulation, and ability to produce high titers of highly infective recombinant virus. Additionally, the wild-type adenovirus is associated with minimal toxicity in humans.

The use of adenovirus-mediated gene therapy to introduce the *p53* gene into tumor cells is an evolving and potentially valuable approach to the treatment of many types of cancers currently resistant to therapeutic intervention. The main purpose of this review is to outline the most recent advances in the preclinical studies, clinical development of adenovirus-mediated *p53* gene therapy, and the challenges that lie ahead for future clinical studies.

Preclinical studies

The efficacy of adenovirus-mediated *p53* gene therapy has been demonstrated in numerous human cancer cell lines and xenograft models including those derived from lung, head and neck, breast, ovary, pancreas, prostate, brain and colorectal cancers [22]. Initial studies using recombinant human adenovirus-containing wild-type *p53* gene under the control of either the Ad2 major late promoter of human cytomegalovirus or the immediate early gene promoter demonstrated that introduction of the wild-type *p53* gene into tumor cells via these recombinant adenoviruses inhibited DNA synthesis in a p53-specific, dose-dependent manner [21]. In this study, injection of recombinant adenovirus encoding the wild-type *p53* gene into the peritumoral space of tumors derived from the $p53^{null}$ NIH-H69 human small-cell lung cancer cell line reduced tumor growth and increased survival time compared with controls. The effect of endogenous mutant *p53* on the ability of wild-type *p53* to suppress tumor cell growth was also assessed in a series of 45 human cell lines that contained either wild-type or mutated p53 protein or no p53 protein [24]. A positive correlation was observed between the percentage of tumor cells that were transduced and the antiproliferative effects of adenoviral *p53* in $p53^{null}$ and $p53^{mut}$ cells. However, infection with adenoviral *p53* had minimal effect on cells expressing wild-type p53. In human xenograft models, adenoviral *p53* gene transfer suppressed tumor growth in $p53^{null}$ and $p53^{mut}$ tumors, including tumors with dominant negative *p53* mutations, and increased survival times.

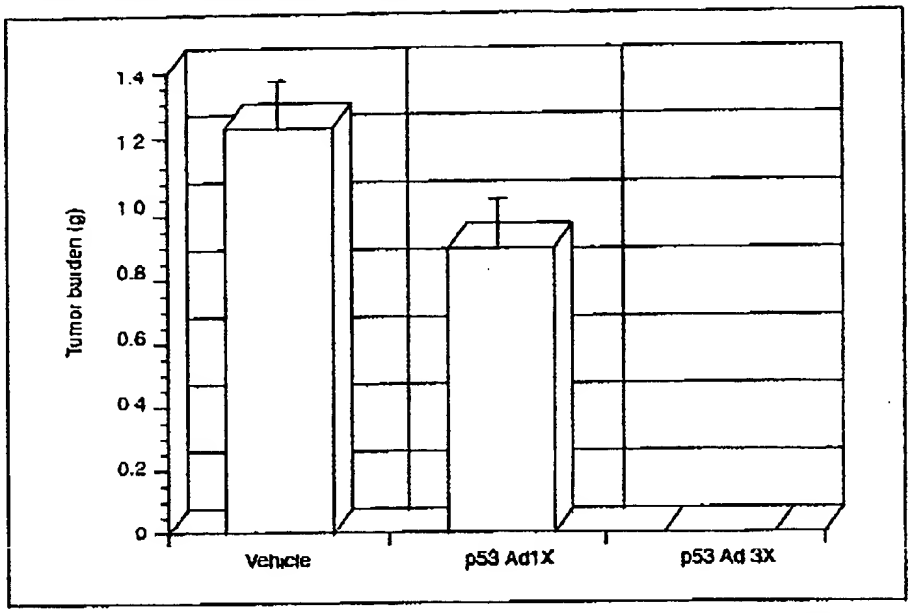
Recently the combination of adenoviral *p53* gene transfer with other modalities and in the sensitization of chemotherapy-resistant disease has been evaluated in a variety of tumor types. A brief review of the results from some of these studies and their clinical applications follows.

Ovarian cancer

Based on the regional nature of the disease and antitumor activity in ovarian xenograft models, adenovirus-mediated *p53* gene therapy is currently being investigated in clinical trials for the treatment of recurrent ovarian cancer [21,25-31]. Mujoo *et al* [28] demonstrated the efficacy of adenovirus-mediated *p53* gene therapy in a highly aggressive ovarian SK-OV-3 xenograft model. In this study, *ex vivo* treatment of SK-OV-3 cells with recombinant adenoviral *p53* prior to infection into nude mice increased survival by more than 50% over control animals. Long-term survival of 166 to 423 days was noted in an intraperitoneal SK-OV-3 xenograft model treated with recombinant adenoviral *p53* [28]. Nielsen *et al* [30] observed similar results in a study evaluating the efficacy of different dosing strategies in the SK-OV-3 xenograft model. Tumor burden was significantly reduced in all mice treated with adenoviral *p53* gene therapy ($P \leq 0.008$). In this study, fractionated doses of adenoviral *p53* had somewhat greater efficacy compared with a single bolus injection (Figure 2) [30]. In contrast, no survival advantage was observed for adenovirus-mediated *p53* gene therapy in human 2774 ovarian xenograft model [31]. It is possible that the type of *p53* mutations and presence of mismatch repair defect in the 2774 cell line may have contributed to the lack of p53-specific response observed *in vivo*.

The antitumor effect of *p53* gene therapy was also observed in human ovarian cancer cells that were resistant to cisplatin [27]. In this study, infection with adenoviral *p53* resulted in a 10-fold increase in sensitivity to cisplatin in the cisplatin-resistant C-1 cell line. Cell cycle analysis revealed that infection with adenoviral *p53* increased the number of cells undergoing apoptosis in cisplatin-resistant cells in comparison with parental cell line. Additionally, in an intraperitoneal C-1 xenograft model, *p53* gene therapy increased survival in more than 50% of the animals, demonstrating that *p53* adenoviral gene therapy may be useful in the treatment of drug-resistant disease. These results have lead to the investigation of intraperitoneal adenoviral *p53* gene therapy for the treatment of platinum and paclitaxel-resistant ovarian cancer.

Figure 2. Efficacy of fractional doses versus single bolus doses of *p53*.



Fractionated dosing versus single bolus injection of adenoviral *p53* in SK-OV-3 ovarian xenograft model. *(Hum Gene Ther Vol 9 No 5, 1998 p681-694. Copyright 1998 Reprinted by permission of Mary Ann Liebert Inc.)*

Pancreatic cancer

Two recent studies revealed the antitumorigenic effects of adenovirus-mediated *p53* gene therapy in human pancreatic cancer both *in vitro* and *in vivo* [32,33]. Although transduction efficiency varied among the cell lines tested, adenovirus-mediated *p53* gene transfer suppressed growth of all human pancreatic tumor cell lines in a dose-dependent manner. A 4-fold increase in apoptotic cells was observed in MiaPaCa-2 cell line at 48 and 72 h following infection [32]. Similar effects were observed in xenografts established from these cell lines after intratumoral injections of adenoviral *p53*.

Bladder cancer

The application of *p53* gene therapy as an alternative treatment for bladder cancer has recently been investigated in an orthotopic model of bladder cancer in rats [34]. In this model, intravesicular administration of adenoviral *p53* resulted in increased p53 expression that corresponded with areas of apoptotic cell death in tumor tissues. Based on preclinical data, there are phase I clinical investigations evaluating intravesicular administration of adenoviral *p53* for the treatment of locally advanced bladder cancer in progress.

Hepatocellular carcinoma and intra-arterial delivery

The potential for intra-arterial delivery of adenovirus-mediated *p53* gene therapy for the treatment of liver malignancies has also been evaluated in a syngenic rat model of hepatocellular carcinoma [35]. For these studies, multifocal tumor nodules were produced in buffalo rats using the McA-RH7777 $p53^{mut}$ hepatocellular cell line. Intrahepatic arterial delivery of adenoviral *p53* increased expression of wild-type p53 and suppressed tumors when compared with untreated or mock-infected animals. Additionally, intrahepatic arterial dosing with adenoviral *p53* decreased systemic exposure to adenovirus compared with intravenous dosing. Based on these results, a phase I dose-escalation study has been initiated to evaluate the safety and potential gene transfer for intra-arterial administration of adenoviral *p53* in patients with colorectal liver metastasis.

Glioblastoma

Mutations and aberrations in the expression of the *p53* gene occur in 30% to 65% of all malignant gliomas, suggesting an early role in the initiation of tumorigenesis. Introduction of *p53* gene into glioma cell lines has been shown to induce apoptosis in tumor cells encoding mutant *p53* gene [36,37]. In these studies, introduction of adenoviral *p53* had a minimal effect on suppressing the growth of glioma cell lines encoding wild-type *p53* gene. However, the results of several recent studies that have investigated the combination of adenovirus-mediated *p53* gene therapy with ionizing radiation indicate there may be a role for *p53* gene therapy as an adjunct to radiation in the treatment of malignant glioma [38-40]. (See 'combination therapy' section.)

Head and neck squamous cell carcinoma

Several studies have demonstrated the antitumor effect of adenovirus-mediated *p53* gene therapy in human head and neck squamous cell carcinoma (HNSCC) cell lines and xenograft models [41-43]. Adenoviral *p53* induced growth arrest and morphological changes consistent with apoptosis in the Tu-138 HNSC cell line and xenograft model [41,42]. In additional studies using a subcutaneous microscopic

residual HNSCC xenograft model that mimics the post-surgical environment of head and neck cancer patients with advanced disease, adenovirus-mediated *p53* gene therapy suppressed tumors, regardless of the *p53* genotype of the tumor cells [43]. These results have led to the initiation of two phase I studies to determine the feasibility and safety of *p53* gene therapy in patients with advanced recurrent HNSCC.

Applications in chemotherapy-resistant breast cancer

Seth *et al* [44] evaluated the cytotoxic effects of adenovirus-mediated *p53* gene therapy in two breast cancer MCF-7 cell lines selected for resistance to adriamycin (MCF-Adr) and mitoxantrone (MCF-Mito). In this study, both MCF-Adr and MCF-Mito cell lines were 20- to 30-times more sensitive to the cytotoxic effects of adenoviral *p53* than parental MCF-7 cell lines. Infection with 3.2 pfu/cell of adenoviral *p53* resulted in a 2-fold reduction in the IC50 of adriamycin in adriamycin-resistant cells. Adenoviral *p53* infection induced apoptosis in both MCF-Adr and MCF-Mito cell lines while parental MCF-7 cell lines failed to undergo apoptosis. Additionally, infection of a mixed population of MCF-Adr and CD34+ cells with adenoviral *p53* selectively inhibited the growth of drug-resistant breast cancer cells and had no effect on CFU-GM colony formation from the CD34+ cells. These data suggest gene therapy may be effective in sensitizing cells to the effects of chemotherapy and also support a role for *p53* gene transfer in purging stem cell products of patients undergoing autologous bone marrow transplantation.

Combination of traditional therapy with adenovirus-mediated *p53* gene therapy

Drug resistance that develops in many different human cancers during initial therapy or relapse has substantial impact on the overall outcome and success of cancer therapy. The loss of functional p53 in different types of tumor cells has been associated with resistance to chemotherapeutic agents [45,46]. The efficacy of combining adenovirus-mediated *p53* gene therapy with chemotherapy has been investigated in a variety of different tumor types, including carcinomas of the lung, ovary, breast and colon [26,29,38,39,47-50].

Combination with cisplatin

Adenovirus-mediated *p53* gene therapy has been demonstrated to increase the sensitivity of a number of different tumor types to the cytotoxic effects of cisplatin. Fujiwara *et al* [19] were among the first to demonstrate that this combination had an additive effect on growth inhibition of the human lung cancer H358 cell line. Nguyen *et al* [51] reported similar results *in vitro* and *in vivo* using the $p53^{-/-}$ human lung cancer H1299 xenograft model where treatment of H1299 cells with low concentrations of cisplatin 48 h before infection with adenoviral *p53* inhibited growth 31% to 60%. A higher level of p53 protein expression and fraction of apoptotic cells was observed in cells treated with this combination compared with cells infected with adenoviral *p53* alone. Systemic administration of cisplatin before, during, or after the intratumoral administration of adenoviral *p53* also resulted in pronounced inhibition of tumor growth in the H1299 xenograft model. The administration of cisplatin before infection with adenoviral *p53* was the most effective *in vivo* dosing schedule. Additionally, a second cycle of gene therapy resulted in greater growth suppression compared with a single cycle of therapy [51]. This combination has also been used for the treatment of non-small-cell lung cancer (NSCLC) [52].

Similarly, Ogawa *et al* [49] demonstrated increased sensitivity to cisplatin cytotoxicity in the $p53^{mut}$ WiDr human colon cancer cell line and xenograft model transduced with adenoviral *p53*. Transduction of WiDr cells with 50 pfu/cell resulted in a high level of p53 expression with no cytotoxic effects. Combination with cisplatin produced an enhanced antitumor effect with highest growth suppression observed at 1 μg/ml of cisplatin. Administration of intraperitoneal cisplatin after intratumoral adenoviral *p53* significantly enhanced growth suppression in WiDr xenografts compared with adenoviral *p53* alone ($P < 0.05$). Kanamori *et al* [50] also noted significant growth suppression of SK-OV-3 cells treated with adenoviral *p53* gene therapy and cisplatin. In this study there was a positive correlation between level of adenoviral *p53* transduction and increased sensitivity of SK-OV-3 cells to cisplatin. Miyake *et al* [53] also noted increased sensitivity of a subcutaneous HT1376 human bladder xenograft model to cisplatin following introduction of adenoviral *p53*. Direct injection of adenoviral *p53* vector into pre-existing tumors, followed by intraperitoneal administration of cisplatin, induced apoptotic destruction of tumors. These findings suggest that the combination of adenovirus-mediated *p53* gene therapy and cisplatin may be an efficient tool for the treatment of cancer.

Combination with paclitaxel

Recently, Nielsen *et al* [29] demonstrated that combination of adenovirus-mediated *p53* gene therapy with paclitaxel increased the sensitivity of human head and neck, ovarian, prostate and breast cancer to the cytotoxic effects of paclitaxel *in vitro* and *in vivo*. In this study, pretreatment of cells with paclitaxel 24 h before exposure to adenoviral *p53* or with both agents simultaneously had either a synergistic or additive effect, depending on the cell line tested. Of interest was the observation that concentrations of paclitaxel, which were lower than that required for microtubule concentration, resulted in a dose-dependent increase in transduction of cells with adenoviral p53 vector. Cell cycle analysis revealed that cellular response to the combination depended on the relative concentrations of the two agents. Higher levels of paclitaxel yielded G2 arrest, while higher levels of adenoviral *p53* resulted in a G0/G1 arrest prior to apoptosis. *In vivo*, combination of paclitaxel and adenoviral *p53* gene therapy produced significant reduction in tumor growth in ovarian (SK-OV-3), prostate (DU-145) and two breast (MDA-MB 468 and MDA-MB 231) xenograft models compared with either treatment alone. These data indicate that combination of paclitaxel and adenoviral *p53* gene therapy is effective in different tumor types.

Combination with IL-2

Putzer *et al* [48] evaluated the efficacy of combined adenoviral gene therapy with p53 and IL-2 expressing vectors to stimulate immune specific antitumor response and tumor regression in a transgenic breast xenograft model. Single intratumoral injection of adenoviral *p53* (1×10^9 *pfu*) and low doses of adenoviral *IL-2* (1.5×10^7 pfu) resulted in 65% reduction in tumor size without toxicity. In

contrast, treatment with either vector alone at the same dose resulted in delayed tumor growth. Tumor regression was associated with long-term immunity, since 50% of mice remained tumor free and were immune to rechallenge with fresh tumor cells. Combination therapy was also associated with development of specific cytolytic T-lymphocyte response compared with either treatment alone.

Combination with 2-methoxyestradiol

Kataoka *et al* [54] evaluated the combination of adenovirus-mediated *p53* gene therapy with 2-methoxyestradiol in human metastatic lung cancer cells *in vivo* as a method for improving the effectiveness of *p53* gene therapy in the treatment of lung metastases. Simultaneous administration of *p53* and 2-methoxyestradiol resulted in a greater than additive reduction, with the lung colony count reduced by 33% compared with control values. These results suggest that the synergistic effect of this combination may have an application in the systemic treatment of lung cancer

Combination with irradiation

The effect of adenovirus-mediated *p53* gene therapy on the radiosensitivity of tumor cells has been the focus of several recent studies [26,38,39,55-57]. Spitz *et al* [55] examined the effect of adenoviral *p53* gene therapy and irradiation on $p53^{mut}$ SW620 colorectal tumor cells *in vitro* and *in vivo*. Transduction of cells with adenoviral *p53* 2 days prior to irradiation with 2 Gy resulted in 50% to 60% reduction in cell survival via apoptosis compared with cells that were mock- or vector-infected prior to irradiation. This combination also produced significant tumor growth suppression in subcutaneous SW620 xenografts pretreated with three consecutive doses of adenoviral *p53* prior to 5 Gy of irradiation ($P < 0.01$). Similar results were observed in a $p53^{mut}$ SK-OV-3 ovarian xenograft model [26]. In this study, intratumoral administration of adenoviral *p53* (10^9 pfu) 2 days before treatment with radiation led to a 45% reduction in tumor size compared with either treatment alone, in mock-infected and untreated controls.

The ability of adenovirus-mediated *p53* gene therapy to sensitize human glioma cells that encode mutant p53 to irradiation has also been evaluated. Introduction of wild-type *p53* into the $p53^{mut}$ human U87MG glioma cell line via adenoviral vector 2 days before exposure to irradiation (9 Gy dose) significantly increased radiation-induced apoptosis compared with mock-infected controls ($P < 0.001$) [38]. Further analysis showed that irradiation of U87MG glioma cells infected with adenoviral *p53* resulted in increased expression of both p53 protein and p21 mRNA levels.

Badie *et al* [39] also investigated the combination of adenovirus-mediated *p53* gene therapy and irradiation in a rat 9L gliosarcoma xenograft model. Stereotactic injection of adenoviral *p53* (10^9 pfu/ml) into pre-existing brain tumors resulted in a modest reduction in tumor volume However, administration before radiation produced a significant (85%) reduction in tumor size compared with control animals ($P < 0.0008$). Moreover, combination therapy improved survival, with 29% (2/7) of animals in the combined treatment group remaining tumor free 2 weeks after treatment. Analysis of brain tissue from surviving animals in the combined treatment group revealed no microscopic evidence of tumor.

Although these results have important implications for improving the treatment of malignant glioma and metastatic brain tumors, further studies in human brain tumor xenograft models with different p53 status are needed to confirm the efficacy of this combination *in vivo*.

p53-Mediated sensitization of HNSCC cells to radiotherapy has also been demonstrated *in vitro* and *in vivo* [56,57] Treatment of radiation resistant JSQ-3 HNSCC cell line with adenoviral *p53* inhibited growth *in vitro* and *in vivo* while having no effect on normal cells. More significantly, introduction of *p53* also resulted in a dose-dependent reduction in the radiation resistance. A single dose of adenoviral *p53* combined with ionizing radiation markedly enhanced radiosensitivity of JSQ-3 xenograft with complete long-term regression of tumors for up to 162 days. These results provide further evidence of the efficacy of this combination and indicate that adenoviral *p53* sensitization of tumors to radiation therapy may significantly reduce the rate of recurrence of certain tumors after radiation treatment.

Clinical studies of adenovirus-mediated *p53* gene therapy

Extensive preclinical studies have evaluated the safety of using replication-deficient type 5 adenoviral vectors encoding wild-type *p53* under the control of the human cytomegalovirus immediate early gene promoter to transfer genes to human cells [21,24]. In these studies, doses of the adenoviral *p53* that are cytotoxic to neoplastic cells had little to no adverse effect on normal cells including fibroblasts, bone marrow cells, and epithelium from the liver, lungs, breast and ovary [41,58,59]. These studies have also demonstrated that intratumoral, intrahepatic and intraperitoneal routes of administration with adenoviral *p53* do not adversely affect surrounding tissues. Similar results have been reported for phase I studies that evaluated the safety and biological effect of adenovirus-mediated *p53* gene therapy in the treatment of primary and metastatic head and neck, lung, liver, colorectal and ovarian tumors (Table 1) [52,60,62-64]. The results of these studies, just now beginning to appear, are summarized below.

Intratumoral delivery

Non-small-cell lung cancer

A phase I single-dose rising study has evaluated adenovirus-mediated p53 gene transfer in advanced NSCLC [52]. Tumors from 15 patients with incurable NSCLC were transduced with one of four doses of single agent adenoviral *p53* ranging from 10^7 to 10^{10} pfu/ml. Adenoviral *p53* was administered as a single bronchoscopic or computed tomography (CT)-guided percutaneous intratumoral injection. The tumors from all patients had high levels of p53 as detected by immunohistochemistry, suggesting mutations in the *p53* gene. Successful gene transfer and expression of exogenous wild-type p53 occurred at higher concentrations of adenoviral *p53* (10^9 pfu/ml) and vector-related sequences were detected in post-treatment biopsies from six patients. Stabilization of tumor growth was achieved in four of these patients and no clinically significant toxicity due to *p53* therapy was observed [52].

Table 1. Completed clinical trials of adenovirus-mediated *p53* gene therapy.

Disease	Adenoviral p53 dose	Response	Reference
NSCLC	10^7 to 10^{10} pfu/ml	Stable disease was achieved in 4/15	[52]
NSCLC	10^8 to 10^{11} pfu with or without iv cisplatin	10% partial response, 61% stable disease, 25% progressive disease, 74% stable disease for CDDP+ adp53	[60]
Advanced recurrent HNSCC	10^9 to 10^{12} pfu	N/A	[62]
HNSCC	10^8 to 10^{11} pfu over 2 weeks to 6.5 months	18% stable disease, 6% partial response, 3% complete response	[63]
Ovarian	7.5×10^{10} to 7.5×10^{12} particles	N/A	[64]

NSCLC = non-small-cell lung cancer, HNSCC = human head and neck squamous cell carcinoma, CDDP = cisplatin (cis-diamminedichloroplatinum(II)).

Additionally, the safety and therapeutic potential of adenoviral *p53* gene therapy with or without cisplatin in patients with advanced NSCLC who failed conventional therapy was evaluated [60,61]. In this study, 28 patients (89% before radiation and 75% before chemotherapy) were treated with or without intravenous cisplatin 3 days prior to bronchoscopic or CT-guided percutaneous intratumoral injection of escalating doses of adenoviral *p53* (10^8 to 10^{11} pfu) [61]. Patients received up to six intratumoral injections of adenoviral *p53* at monthly intervals. A total of 84 courses were administered, with 56 doses (67%) being repeat injections. The majority of patients (68%) received up to three courses of adenoviral *p53*, while 11%, 7% and 14% patients received four, five and six courses of adenoviral *p53*, respectively. Adenoviral *p53* was well-tolerated and produced little toxicity. Vector-related sequences were detected in post-treatment biopsies. Of the 25 patients evaluable for tumor response, two achieved a partial response, 16 demonstrated stable disease and seven progressed after treatment with adenoviral *p53* alone. Transient local control of disease ranged from 2 to 14 months, and more than 50% reduction in tumor size was observed in two patients who received six courses of adenoviral *p53* [61]. A cohort of nine additional patients received adenoviral *p53* in conjunction with cisplatin given at a dose of 80 mg/m^2 intravenously over 2 h, 3 days before injection with adenoviral *p53* [60]. Stabilization of disease was slightly higher (74% CDDP+ adp53 verses 61% for adp53 alone) in this cohort of patients compared with those who received adenoviral *p53* alone. An analysis of factors that affect disease progression revealed that higher doses of adenoviral *p53*, concomitant cisplatin therapy, and increased apoptosis as demonstrated by *in situ* DNA nick end labeling staining of tumor specimens were associated with enhanced time to progression [61]. These encouraging results have precipitated the design of a phase II study to assess the efficacy of adenovirus-mediated *p53* gene therapy in combination with radiation.

Advanced recurrent head and neck squamous cell carcinoma

Two phase I clinical studies have evaluated the safety and biological activity of adenovirus-mediated *p53* gene therapy in patients with resectable and non-resectable advanced recurrent HNSCC [62,63]. In one study, HNSCC tumors from 25 patients were transduced with adenoviral *p53* at doses ranging from 10^9 to 10^{12} pfu [62]. A single injection of either 7.5×10^9 pfu, 7.5×10^{10} pfu or 7.5×10^{11} pfu was administered to three groups of three patients each, respectively. Multiple injections of either 7.5×10^{11} pfu or 1.5×10^{12} pfu were administered to six patients and 10 patients, respectively. Of the patients who received multiple injections, three patients at the 7.5×10^{11} pfu dosage level and six patients at the 1.5×10^{12} pfu dosage level received chemotherapy concurrently. Successful transduction of tumor was observed in four of 10 tumors examined and response to therapy was observed in one patient [62].

Clayman *et al* [63] also evaluated the safety and therapeutic potential of adenovirus-mediated *p53* gene therapy in patients with resectable and non-resectable advanced recurrent HNSCC. In this study, 33 patients received multiple intratumoral doses of adenoviral *p53* alone ranging from 10^8 pfu to 10^{11} pfu over a course of 2 weeks to 6.5 months. In patients with non-resectable tumors, objective tumor regression of > 50% was observed in two patients, while stabilization of disease for up to 3.5 months was achieved in another six patients. Additionally, one patient with resectable disease was considered to have achieved a complete pathological response in that no viable tumor was found in the completely resected specimen [63]. Based on these results, a phase II study is currently examining the effect of adenoviral *p53* gene therapy on response rate, duration of response, time to progression, overall survival, and quality of life in patients with recurrent HNSCC.

Intraperitoneal delivery: Recurrent ovarian cancer

The efficacy of intraperitoneal *p53* gene therapy alone or in combination with chemotherapy was evaluated in 37 women with advanced ovarian cancer that was refractory to conventional therapy [64]. Patients received a single dose of adenoviral *p53* ranging from 7.5×10^{10} to 7.5×10^{12} particle number (pn). Expression of transgene was detected by reverse transcriptase polymerase chain reaction (RT-PCR) in some tumor samples at the lowest dose level and consistently at the 7.5×10^{11} pn dose level and above. Once the safety of single injection was established, patients received multiple daily doses of adenoviral *p53* ranging from 7.5×10^{12} to 7.5×10^{13} pn concurrently with chemotherapy every 21 to 28 days. The highest dose evaluated was 7.5×10^{13} pn daily for 5 days concurrently

with chemotherapy and was well-tolerated with mild analgesic and antipyretic prophylaxis [64]. The maximum tolerated dose for this study was not identified. Overall the intraperitoneal administration of adenoviral *p53* gene therapy was well-tolerated [64]. Further phase I studies continue to evaluate intraperitoneal *p53* gene therapy alone or in combination with other chemotherapeutic agents for the treatment of advanced, recurrent, or persistent ovarian cancer, as well as in the treatment of platinum- and paclitaxel-resistant ovarian cancer.

Intrahepatic arterial delivery: Colorectal liver metastasis and hepatocellular carcinoma

A phase I dose-escalation study has been initiated to evaluate the safety and gene transfer for intra-arterial administration of adenoviral *p53* in patients with colorectal liver metastasis and hepatocellular carcinoma [65]. Currently 16 patients with immunohistochemical evidence of *p53* mutation have been enrolled. Cohorts of three patients each have received doses of adenoviral *p53* beginning at 7.5×10^7 pfu and escalating to 7.5×10^{10} pfu. The maximum tolerated dose was defined at 2.5×10^{11} pfu. Expression of transgene has been detected in the tumor by RT-PCR at the 2.5×10^{11} pfu dose level [65]. Intra-arterial administration of adenoviral *p53* gene therapy has been well-tolerated and evidence of dose-limiting toxicity has been observed at the highest dose level [Horowitz JA, unpublished data]. Evaluation of dose-escalation of adenoviral *p53* combined with chemotherapy continues.

The results of these phase I studies demonstrate that intratumoral, intraperitoneal and intrahepatic arterial delivery of adenoviral *p53* gene therapy is well-tolerated and results in the successful expression of wild-type *p53* into various tumor types. Anecdotal reports of clinical responses support further investigation. Several additional studies have been initiated to evaluate the use of adenovirus-mediated *p53* gene therapy in the treatment of other malignancies, including bladder cancer and malignant glioma (Table 2). Phase II studies have been initiated to further evaluate adenoviral *p53* gene therapy in the treatment of HNSCC, NSCLC, and ovarian and colorectal cancers.

Issues for adenovirus-mediated *p53* gene therapy

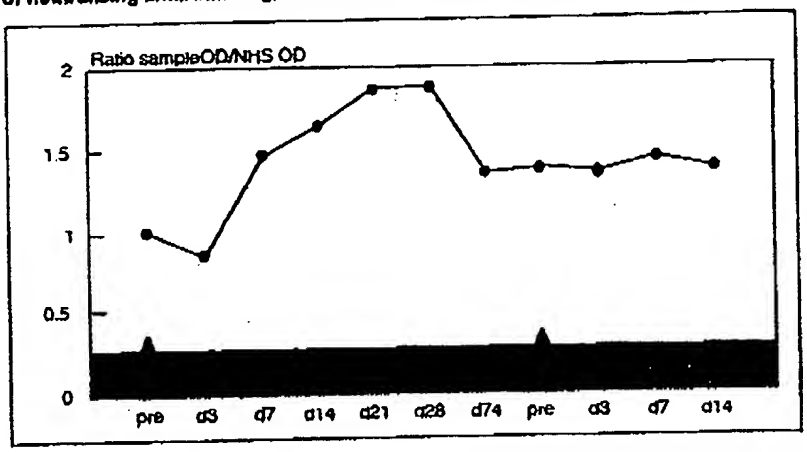
The above preclinical and clinical phase I studies confirm that the exogenous transgene can be expressed in tumors by various routes of administration, therefore confirming the proof of concept. The largest obstacle to human gene therapy is the delivery of the transgene to the tumor site. This issue affects all delivery systems identified to date. The issue specific to adenoviruses is the rapid clearance of the vector and induction of host immune response to the adenovirus. This may result in the requirement for higher doses of adenovirus to overcome this obstacle, or the need for the vector to be delivered by intratumoral or regional intrahepatic artery or intraperitoneal route.

Most advanced malignancies are systemic in nature, and delivery by intratumoral or regional routes place limitations on gene therapy. The development of alternative delivery systems, and means by which this delivery system can be administered systemically, are under investigation. Until such alternatives are available, the addition of systemic chemotherapy enhances this form of novel therapy for advanced disease. It is encouraging that the individual patient's pre-existing immunity to adenovirus has not precluded expression of the exogenous transgene (Figure 3) [52] and that the safety profile of these agents when combined with chemotherapy are acceptable. The impact that this immunity has on the dose intensity, however, can be inferred. In addition, it is encouraging that the wide tissue tropism of the adenoviruses has not resulted in undue or unmanageable safety issues, despite published preclinical models where hepatic toxicity was observed [30,66,67].

Table 2. Ongoing clinical trials of adenovirus-mediated *p53* gene therapy.

Phase	Description
Phase I dose-escalation study	Intravesicular administration of adenoviral *p53* for treatment of locally advanced and metastatic bladder cancer
Phase I multicenter dose-escalation study	Intratumoral stereotactic injection of adenoviral *p53* for treatment of recurrent malignant glioma
Phase I dose-escalation study	Intraperitoneal delivery of adenoviral p53 for treatment of advanced, recurrent, or persistent ovarian cancer
Phase I dose-escalation study	Intraperitoneal delivery of adenoviral *p53* for treatment of platinum- and paclitaxel-resistant ovarian cancer
Phase I pilot dose-escalation study	Delivery of adenoviral *p53* by bronchoalveolar lavage for treatment of bronchoalveolar cell lung cancer
Phase I dose-escalation study	Percutaneous injections of adenoviral *p53* for hepatocellular carcinoma
Phase I dose-escalation study	Intra-arterial delivery of adenoviral *p53* for treatment of primary and metastatic tumors of the liver
Phase I dose-escalation study	Combination of chemotherapy with single and multiple intraperitoneal injections adenoviral *p53* for treatment of peritoneal carcinomas
Phase II study	Recurrent squamous cell carcinoma of the head and neck
Phase II/III	Intraperitoneal delivery of *p53* adenovirus for treatment of newly diagnosed ovarian cancer
Phase II	Intra-arterial delivery of *p53* adenovirus for treatment of colon cancer metastatic to the liver

Figure 3. Development of neutralizing antibodies against adenoviral vector following the first treatment with adenoviral *p53*.



Course of anti-adenoviral p53 antibodies in a single patient receiving two doses of adenoviral *p53*. The black triangles show the time points of treatment. The shaded area highlights the negative threshold of 0.28 (Hum Gene Ther Vol 9 No 14, 1998 p2675-2082 Copyright 1998 Reprinted by permission of Mary Ann Liebert Inc.)

Conclusion

The results of preclinical and clinical studies have demonstrated that adenovirus-mediated gene therapy is a safe and efficient method for the introduction of the wild-type *p53* gene in a variety of human cancers. It is clear that the treatment of tumor cells with adenoviral *p53* causes tumor regression. Evidence from *in vitro* and *in vivo* studies indicate that adenovirus-mediated *p53* gene therapy potentiates the cytotoxicity of both chemotherapeutic agents and radiation therapy in a variety of cancers. Results from initial clinical studies have confirmed the safety of adenovirus-mediated *p53* gene therapy. Future studies will be needed to determine the efficacy of adenovirus-mediated *p53* gene therapy and its role in the management of cancer patients. Such studies are underway.

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Successful Adenovirus-Mediated Wild-Type *p53* Gene Transfer in Patients With Bladder Cancer by Intravesical Vector Instillation

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Purpose: To study safety, feasibility, and biologic activity of adenovirus-mediated *p53* gene transfer in patients with bladder cancer.

Patients and Methods: Twelve patients with histologically confirmed bladder cancer scheduled for cystectomy were treated on day 1 with a single intratumoral injection of SCH 58500 (rAd/p53) at cystoscopy at one dose level (7.5×10^{11} particles) or a single intravesical instillation of SCH 58500 with a transduction-enhancing agent (Big CHAP) at three dose levels (7.5×10^{11} to 7.5×10^{13} particles). Cystectomies were performed in 11 patients on day 3, and transgene expression, vector distribution, and biologic markers of transgene activity were assessed by molecular and immunohistochemical methods in tumors and normal bladder samples.

Results: Specific transgene expression was detected in tissues from seven of eight assessable patients treated with intravesical instillation of SCH 58500 but in none of three assessable patients treated with intratumoral injection of SCH 58500. Induction of RNA and protein expression of the p53 target gene *p21/WAF1* was demonstrated in samples from patients treated with SCH 58500 instillation at higher dose levels. Distribution studies after intravesical instillation of SCH 58500 revealed both high transduction efficacy and vector penetration throughout the whole urothelium and into submucosal tumor cells. No dose-limiting toxicity was observed, and side effects were local and of transient nature.

Conclusion: Intravesical instillation of SCH 58500 combined with a transduction-enhancing agent is safe, feasible, and biologically active in patients with bladder cancer. Studies to evaluate the clinical efficacy of this treatment in patients with localized high-risk bladder cancer are warranted.

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AN ESTIMATED 261,000 new cases of bladder cancer are diagnosed worldwide per year. Bladder cancer is prevalent in the developed countries, where it affects mainly men and is frequently associated with a history of tobacco smoking or some occupational exposures, and in Northern Africa and Western Asia, where it is related to endemic infection with the parasite *Schistosoma mansonii*.[1] In the Western world, 70% to 80% of patients present with superficial bladder tumors, which can be treated with transurethral resection.[2,3] However, patients with less differentiated, large or multilocular bladder tumors as well as patients with carcinoma in situ or stage I bladder cancer are at high risk for tumor recurrence and development of muscle-invasive disease or distant metastases.[4,5] Treatment strategies for such high-risk patients include local resection with close surveillance,[2] local resection and intravesical therapy using bacillus Calmette-Guerin or cytotoxic agents,[6-8] or radical cystectomy with urinary diversion or reconstructive surgery.[9,10] Radical cystectomy provides optimal control of the bladder tumor, but at the price of organ loss. Intravesical and systemic medical therapies have substantial toxicities and bear the risk of local recurrence or tumor progression. Thus, new bladder-preserving treatment options for high-risk bladder cancer are required.

Mutations of the *p53* tumor suppressor gene are the most common genetic alteration in human cancers.[11] The role of p53 in the prevention of oncogenic transformation, maintenance of genetic stability, and sensitivity to commonly used cancer treatments is well established.[12,13] In some but not all studies, nuclear accumulation of p53 as an indicator for mutations in the p53 DNA binding domain was associated with an adverse prognosis in patients with bladder cancer.[14-17] Hence, somatic gene transfer of the *p53* tumor suppressor is an attractive new treatment modality for malignant bladder tumors. Preclinical cancer models have

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demonstrated that the expression of *p53* by viral or nonviral gene transfer technology effectively induced apoptosis or sensitized cancer cells to drug- or radiation-induced cell death.[18] These results have fostered the translation of *p53* gene therapy into early clinical studies, which were conducted in patients with advanced lung, head and neck, ovarian, or liver cancers.[19-23] Using intratumoral injection of adenoviral[20-22,24] or retroviral[19] *p53* expression vectors, local transgene expression[20,22] and evidence for local tumor regressions and induction of apoptosis[19,24] were reported from several phase I and pilot studies. However, the only controlled phase II study in patients with newly diagnosed advanced non–small-cell lung cancer (NSCLC) failed to demonstrate a significant clinical benefit from local *p53* gene transfer by intratumoral vector injection in combination with an effective first-line chemotherapy.[25] One reason for this apparent clinical inactivity might be insufficient gene delivery and transduction after intratumoral injection of adenoviral *p53* expression vectors. Systematic studies of these important parameters, however, are absent in cancer patients.

One way to overcome the potential limitations of the intratumoral injection approach is the instillation of high-vector doses into cavitary organs, such as the pleural space,[26] peritoneal cavity, or bladder. This should allow a homogeneous vector distribution along the tumor surfaces, as opposed to a vector distribution along the track of an injection needle. Preclinical studies have demonstrated the feasibility of this approach and have highlighted the importance of the addition of transduction-enhancing agents to maximize transgene expression in the bladder.[27,28]

To address this hypothesis, a study of safety, feasibility, and biologic activity of an intravesical instillation or an intratumoral injection of an adenoviral expression vector encoding wild-type p53 (SCH 58500) was conducted in patients with invasive bladder cancer. To allow assessment of vector distribution, transgene expression, and induction of p53 target genes or additional markers of biologic activity after the study treatment, only patients scheduled for radical cystectomy were enrolled onto this trial, enabling extensive tissue sampling for these analyses.

PATIENTS AND METHODS

Patients

Adult patients with histologically confirmed, muscle-invasive bladder cancer and indication for radical cystectomy were eligible for enrollment. Additional inclusion criteria were a life expectancy of at least 3 months, a Karnofsky performance score of at least 70%, and the absence of any clinical or laboratory evidence (WBC count $\geq$ 3,000/μL, absolute neutrophil count $\geq$ 1,000/μL, platelet count $\geq$ 100,000/μL, creatinine $<$ 1.5 mg/dL, bilirubin $<$ 1.5 mg/dL, AST and ALT $<$ 1.5 times the upper limit of normal, and prothrombin and partial thromboplastin times within normal limits) for dysfunction of the hematopoietic, liver, renal, or coagulation systems. An interval of at least 4 weeks between prior chemotherapy, radiation, or major surgery was mandatory. Pregnant or nursing women, fertile women not practicing medically accepted contraception, patients with uncontrolled serious bacterial, fungal, or viral infections, human immunodeficiency virus–positive patients, and immunosuppressed patients were not eligible. Molecular or immunohistochemical evidence for an intratumoral *p53* mutation was not required for eligibility. All patients provided written informed consent. After written informed consent, control tissue samples were obtained from patients with advanced bladder cancer or patients with nonmalignant bladder disease treated by cystectomy.

Study Design

This was an open-label, single-center, phase I dose-escalation study of a single intratumoral injection (part A) or a single intravesical instillation (part B) of SCH 58500 (rAd/p53). Three patients were treated at each dose level, and dose escalation proceeded if no dose-limiting toxicity was observed. A dose-limiting toxicity was defined as any World Health Organization (WHO) grade 4 toxicity or any WHO grade 3 toxicity lasting more than 1 week. Adverse events that were clearly related to cystoscopy, catheter placement, cystectomy, or palliative treatment to the tumor were not considered dose-limiting. The protocol was approved by the local ethics committee (Bezirksärztekammer Rheinhessen) and the National Regulatory Office (Kommission Somatische Gentherapie der Bundesärztekammer). The study was conducted according to the Declaration of Helsinki (amended version, Hong Kong, 1989) and following the principles of good clinical practice.

Study Treatments

SCH 58500 is a replication-defective recombinant adenoviral vector encoding the complete human wild-type *p53* cDNA.[20,29] Doses were 7.5×10^{11} particles in level 1, 7.5×10^{12} particles in level 2, and 7.5×10^{13} particles in level 3. Patients treated in part A received a single intratumoral injection of 1 mL SCH 58500 in a standard saline-based solution[20] at cystoscopy on day 1. Patients treated in part B received a single intravesical instillation (total volume, 120 mL) of SCH 58500 in 20 mg/mL solution of Big CHAP, a transduction-enhancing agent,[28] through a transurethral catheter on day 1. After instillation, the catheters were blocked to allow a contact time of 60 minutes, followed by release of the catheter and extensive bladder irrigation with saline. During the course of the study, the vector instillation was divided into two sequential administrations of 50% of the vector dose each. The planned contact time for each half dose was 30 minutes; the second instillation immediately followed the release of the first dose. After treatment, all patients were hospitalized in single rooms in a biosafety environment at the study center for at least 24 hours or until adenovirus shedding was no longer detectable. Approximately 48 hours after vector administration (day 3), all patients underwent routinely scheduled radical cystectomies, which were not part of the study treatment.

Study End Points

The primary objective of this study was to assess the safety, feasibility, and toxicity of a single dose of SCH 58500 administered by intratumoral injection (part A) or by intravesical instillation (part B) in patients with invasive bladder cancer. Secondary end points were to

Table 1. Sequences of the Oligonucleotide Primers and Probes Used in Real-Time RT-PCR Assays

Target Gene	Function	Sequence	Expected PCR Product Size (bp)
p21/WAF1	Forward primer	TGGAGACTCTCAGGGTCGAAA	65
	Reverse primer	GGCGTTTGGAGTGGTAGAAATC	
	Probe	CGGCGGCAGACCAGCATGAC	
SCH 58500	Forward primer	AACGGTACTCCGCCACC	94
DNA and RNA	Reverse primer	CGTGTCACCGTCGTGGA	
	Probe	CAGCTGCTCGAGAGGTTTTCCGATCC	
GAPDH	Forward primer	GAAGGTGAAGGTCGGAGTC	226
	Reverse primer	GAAGATGGTGATGGGATTTC	
	Probe	CAAGCTTCCCGTTCTCAGCC	

NOTE. All of the probes were labeled with the reporter signal FAM and TAMRA as the quencher.

assess vector distribution in normal and malignant bladder tissue, transgene expression, and markers of biologic activity in samples obtained at cystectomy.

Clinical Monitoring

Patients were closely monitored for adverse events for the first 7 days after study treatment. After hospital discharge, the patients were followed bimonthly for 1 year at the study center. The monitoring for the first 7 days after treatment included assessment of clinical symptoms, physical examination, monitoring of vital signs, Karnofsky index, concomitant medication, and recording of adverse events. Hematology, serum chemistry, and urinalysis were performed before treatment and on days 1, 2, 4, and 6 and during follow-up visits.

Virology Studies

Adenovirus shedding was monitored in urine, stool, or rectal swab specimens by means of a qualitative enzyme-linked immunosorbent assay (ELISA) before treatment on days 2 and 3 and until no adenovirus shedding was detectable.[20] In addition, urine samples were collected at multiple time points after study treatment and were examined for the presence of infectious adenoviruses by a flow cytometry–based infectivity assay.[30]

Detection of SCH 58500 DNA and Expression of Transgenic p53, p21/WAF1, *and the Coxsackie and Adenovirus Receptors*

SCH 58500 virus DNA, vector-specific transgene expression, p53 target gene *p21/WAF1* expression,[31,32] and Coxsackie and adenovirus receptor (CAR) expression were assessed in tumor samples and normal bladder tissue obtained at cystectomy by reverse transcriptase polymerase chain reaction (RT-PCR), as described previously,[20] and quantitative real-time PCR,[33,34] as described previously.[35] In brief, DNA and RNA were coextracted from frozen bladder samples using Trireagent (Molecular Research Center, Cincinnati, OH). Extracted RNA was DNased, and PCR was performed to ensure no DNA contamination. Real-time quantitative PCR and RT-PCR were performed using the ABI 7700 sequence detector (Applied Biosystems, Foster City, CA). The *GAPDH* gene was used as an internal control to assess the quality of assay samples. Gene expression results were expressed as number of copies per 1,000 copies of *GAPDH*. SCH 58500 DNA was quantified by comparison to viral DNA extracted from purified SCH 58500 virus (Qiagen, Valencia, CA). cRNAs were used as standards to quantify *p53*, *p21*, and *GAPDH* gene expression. The sequences of the oligonucleotide primers and probes are listed in Table 1. Primers for SCH 58500 gene and its expression were designed specifically to amplify SCH 58500 but not the human *p53* gene. Whenever possible, assays were performed on at least two different samples of tumor or nontumor tissue per patient. Bladder tissue samples obtained from patients with advanced bladder cancer, not treated with SCH 58500 served as negative controls. A cutoff level for positive real time PCR samples was set as the detection of at least 10 copies per reaction.[35]

Analysis of Tissue Sections

Localization of SCH 58500 was assessed using a direct in situ PCR method.[36] Formalin-fixed paraffin-embedded tissues were cut into 5-μm sections, placed on in situ PCR slides, and baked for 2 to 3 hours at 60°C on a slide hot plate. The slides were washed in xylene to remove the paraffin, followed by an incubation with 0.02 N HCl and digestion with 2.5 μg/mL proteinase K (Qiagen) at 37°C for 30 minutes. The endogenous alkaline phosphatase activity was eliminated by incubating the slides in ice-cold 20% (vol/vol) acetic acid. Slides were dehydrated in graded alcohols and rehydrated in 45 μL of PCR master mix containing 1 μmol/L of each dinitro-phenyl (DNP)-labeled primer, 200 μmol/L of each dNTP, 2.5 mmol/L magnesium chloride, and 10 units of AmpliTaq DNA polymerase (Applied Biosystems). Primers were designed to amplify a SCH 58500-specific sequence located between the cytomegalovirus promoter (5'-CGTGTCAC-CGTCGTGGA-3') and the upstream *p53* cDNA (5'-CCACTGCT-TACTGGCTTATCGAAAT-3'). This primer selection prevents the amplification of genomic *p53* DNA.[29] Reactions were performed in a Perkin Elmer Gene Amp In Situ PCR System 1000 (Applied Biosystems) programmed for one cycle of denaturation at 95°C for 5 minutes and annealing at 55°C for 90 seconds, followed by 34 cycles of 94°C for 30 seconds and 55°C for 90 seconds. After completion of the PCR, slides were washed two times with standard saline citrate (0.3 mol/L NaCl and 0.03 mol/L sodium citrate) and blocked with casein solution (Vector, Burlingame, CA). For tissue sections, the DNP molecules incorporated into the PCR amplicons were detected using an anti-DNP antibody conjugated with alkaline phosphatase (Applied Biosystems). The sections were stained using the alkaline phosphatase substrate NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) (Boehringer Mannheim, Germany) and then counterstained with Nuclear Fast Red (Vector). As a negative control, each section was processed, but the PCR reaction was performed without AmpliTaqDNA polymerase. Samples from rat bladders instilled with SCH 58500 or a *β-galactosidase*–expressing adenoviral vector (Ad5.β-gal) served as positive and negative controls.

Table 2. Patient Demographics, Tumor Stages, Histologies, and Transgene Expression After SCH 58500 Treatment

Patient No.	Age (years)	Sex	Histology	p53	Stage	Study Group	RT-PCR
001	68	Male	TCC	0	pT4aN2M0 G4	A1	–
002	37	Female	SCC	2	pT3bN0M0 G2-3	A1	–
003	64	Male	TCC	1	pTaN0M0 G2-3	A1	–
004	69	Male	TCC	3	pT1N0M0 G2	B1	+
005	69	Male	TCC	1	pT3aN0M0 G2	B1	+
006	73	Male	TCC	0	pT3aN0M0 G2	B1	+
007	69	Male	TCC	1	pTisN0M0 G3	B2	+
008	69	Male	SCC	3	pT3aN0M0	B2	–
009	70	Male	TCC	0	pT1N0M0 G3	B2	+
010	60	Female	TCC	1	pT1N1M1	B3	ND
011	84	Male	TCC	1	pT2bN1M0 G3	B3	+
012	82	Male	TCC	2	pT4N0M0 G3	B3	+

Abbreviations: TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; Stage, tumor stage according to tumor-node-metastasis classification; p53, immunohistochemical detection of nuclear p53 expression in baseline tumor biopsies in < 10% of tumor cells = 0, 11%-25% of tumor cells = 1, 26%-50% of tumor cells = 2, > 50% of tumor cells = 3; A1, intratumoral injection (part A), dose level 1 (7.5×10^{11} particles); B1, intravesical instillation (part B), dose level 1 (7.5×10^{11} particles); B2, intravesical instillation, dose level 2 (7.5×10^{12} particles); B3, intravesical instillation, dose level 3 (7.5×10^{13} particles); RT-PCR, positive (+) or negative (–) expression of vector-specific *p53* RNA as detected by RT-PCR analysis of samples obtained at cystectomy; ND, not determined (no cystectomy performed).

The protein expression of p53, *p21/WAF1*, apoptosis-related and cell cycle–related genes, and CAR was assessed by immunohistochemistry in formalin-fixed paraffin-embedded tissue sections. Primary antibodies against p53 (M7001, Dako Diagnostika, Hamburg, Germany), *p21/WAF1* (M7202, Dako), Bcl-2 (M0887, Dako), Bak (AM04, Calbiochem, San Diego, CA), Bax (Ab-1/PC66, Calbiochem), MIB1 (dia 505, Dianova, Hamburg, Germany), and CAR (a gift from Dr Robert W. Finberg, Dana-Farber Cancer Institute, Boston, MA[37,38]) were used. Apoptotic cells were visualized by microscopy following the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) method[39] and by means of laser scanning cytometry, as previously described.[40] Normal bladder and tumor tissue samples from patients not treated with SCH 58500 served as controls.

RESULTS

Enrollment and Treatments

Twelve patients from a single center were enrolled onto the study. Baseline characteristics and histologies of the study patients are listed in Table 2. Three patients were treated at dose level 1 in part A (intratumoral injection) of the study. No additional dose escalation was performed in part A. Nine patients were treated at three different dose levels in part B (intravesical instillation). Eleven patients underwent radical cystectomies after study treatment. In one patient, the tumor was determined to be unresectable with curative intent at laparotomy. Thus, tumor samples for assessment of the secondary end points were obtained from 11 patients treated with SCH 58500 at three dose levels.

Toxicity

Postoperatively, one patient treated in part A suffered from WHO grade 1 fatigue. No toxicities were observed in the other two patients treated with intratumoral injection at cystoscopy. The predominant toxicities observed in patients treated in part B of the study were urethral and vesical burning, which reached WHO grade 2 in two patients and WHO grade 3 in another two patients. In addition, one patient each experienced WHO grade 2 and grade 3 abdominal pain. These symptoms were relieved in two patients treated at dose level 1 by a reduction of the contact time, for which the transurethral catheters were clamped. Hence, for patients treated at dose levels 2 and 3, the treatment was administered in two sequential 30-minute sessions. Additionally, patients treated at dose level 3 were premedicated with 50 mg of pethidine and 20 mg of butylscopolamine. Despite these modifications, the planned contact time had to be reduced by several minutes in three patients treated at dose level 2 and in one patient treated at dose level 3. All symptoms resolved immediately after release of the transurethral catheter and bladder irrigation with saline. No fever, chills, or other signs of systemic toxicity were observed in patients treated in part B. No alterations of laboratory parameters, including liver enzymes and bilirubin, were detected before surgery on day 3. Three patients were hospitalized because of fever of unknown origin within 4 to 6 weeks after surgery and quickly recovered under treatment with broad-spectrum antibiotics. In one of these patients, a methicillin-resistant *Staphylococcus aureus* was isolated from a catheter. Thus, even at the highest dose level of 7.5×10^{13} particles SCH 58500 administered by intravesical instillation, no dose-limiting toxicities were observed.

Transgene Expression and Biologic Activity

In two of three assessable patients treated in part A (intratumoral injection), vector DNA was found by PCR

Table 3. Induction of p21/WAF1 RNA and Protein Expression After Intravesical SCH 58500 Treatment

Patient No.	Study Group	Normal Bladder Tissue		Tumor Tissue	
		p21 RNA	p21 IHC	p21 RNA	p21 IHC
Controls	—	1.08 ± 1.8	—	0.26 ± 0.38	—
004	B1	3.5 ± 2.2	0/0	0.92 ± 1.12	0/2
005	B1	2.47	0/0	1.16 ± 1.37	0/2
006	B1	.57 ± 0.4	0/0	1.16 ± 1.43	0/0
007	B2	2.62	0/0	0.32 ± 0.29	0/0
008	B2	ND	0/0	0.32 ± 0.29	0/0
009	B2	4.59	0/0	0.61	0/0
010	B3	ND	ND	ND	ND
011	B3	1.29 ± .42	0/0	10.4 ± 20.69	0/2
012	B3	2.66 ± 1.56	0/0	3.99 ± 4.7	0/1

Abbreviation: ND denotes not determined (insufficient sampling or no cystectomy performed).

NOTE. Tissue samples from bladder tumors and normal bladder tissue obtained at cystectomy were examined by real-time RT-PCR (p21 RNA) and immunohistochemistry (p21 IHC). Normal bladder samples from four patients and tumor samples from five patients not treated with SCH 58500 served as controls for real-time RT-PCR (Controls). Results from real-time RT-PCR are expressed as mean ± SD × 10,000 copies normalized to 1,000 copies *GAPDH* RNA. Results from immunohistochemistry are presented as nuclear expression of p21/WAF1 in biopsies before and after SCH 58500 treatment (< 10% of tumor cells = 0; 11%-25% of tumor cells = 1; 26%-50% of tumor cells = 2; > 50% of tumor cells = 3).

analysis of posttreatment tumor samples (not shown). However, no transgene expression as assessed by RT-PCR analysis of vector-specific *p53* expression was detected after intratumoral injection of SCH 58500 at cystoscopy (Table 2). In contrast, vector-specific *p53* transgene expression was found by RT-PCR analyses of tissue samples from seven of eight assessable patients treated with intravesical instillation of SCH 58500 (Table 2).

To address whether the *p53* transgene expression translated into biologic activity, we determined the quantitative expression of the p53 target gene *p21/WAF1* by real-time RT-PCR analysis of tumor and normal bladder samples from patients treated with intravesical instillation of SCH 58500 or untreated control patients. The *p21/WAF1* expression in tumor samples from untreated control patients was lower than in normal bladder samples (Table 3). Assaying nontumor bladder samples from patients treated with SCH 58500 instillation, moderate changes in *p21/WAF1* expression were detected when compared with untreated controls (Table 3). However, in tumor samples from patients treated at the highest dose level of 7.5×10^{13} particles SCH 58500 *p21/WAF1*, expression was increased up to 40-fold compared with control tumor samples from patients not receiving gene therapy (Table 3). Immunohistochemical analyses revealed an increased *p21/WAF1* protein expression after SCH 58500 treatment in tumor tissues but not in normal bladder samples from four patients with undetectable or low *p21/WAF1* protein expression at baseline (Table 3). No significant correlation between transgene expression, *p21/WAF1* induction, and CAR expression, as determined by RT-PCR analysis and immunohistochemistry, could be established. However, the CAR expression detected by immunohistochemistry exhibited a considerable heterogeneity among tumors from different patients as well as among different regions of the same tumor (not shown). Immunohistochemical analyses of p53 expression or expression of additional apoptosis-related or cell cycle–related genes revealed no consistent changes in relation to SCH 58500 treatment. Moreover, we failed to detect a significant induction of apoptosis as assessed by TUNEL staining and microscopy or laser scanning microscopy in samples taken at cystectomy approximately 48 hours after SCH 58500 treatment (not shown).

Taken together, these data demonstrate that a detectable p53 transgene expression in bladder tumors can be achieved by intravesical instillation of SCH 58500 in combination with a transduction-enhancing agent. At the highest dose level of 7.5×10^{13} particles SCH 58500, evidence for biologic activity in terms of RNA and protein expression of the p53 target gene *p21/WAF1* was obtained.

Vector Distribution

Using quantitative real-time PCR, SCH 58500 DNA copies were detected in normal bladder and tumor samples from patients treated with intravesical instillation in a dose-dependent manner, whereas no SCH 58500 DNA was found in samples from control patients not treated with SCH 58500 (Fig 1). The demonstration of vector DNA or transgene expression in tissue homogenates does not provide information regarding the transduction efficacy or the vector penetration. Therefore, tissue sections from patients treated in part B were analyzed by in situ PCR, revealing a strong vector-specific signal throughout the whole urothelium (Fig 2). Moreover, SCH 58500 DNA was also detected

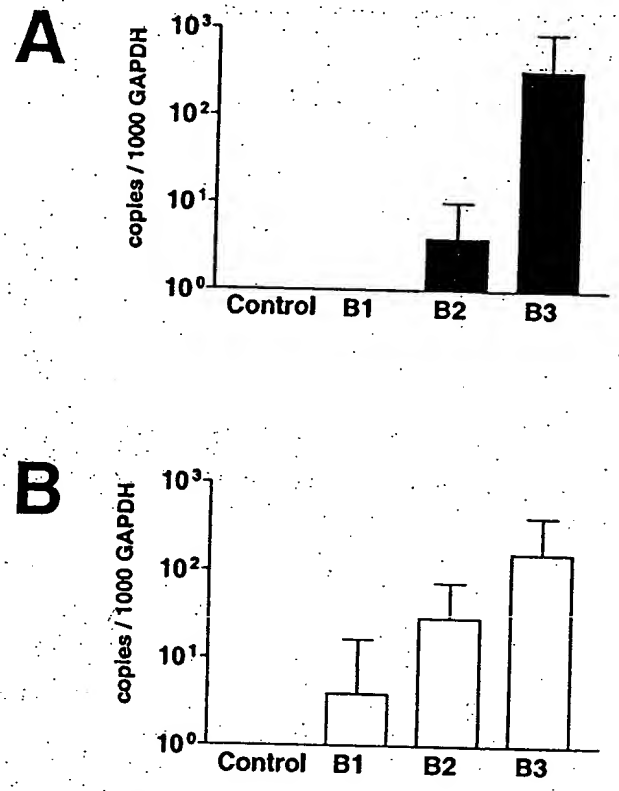

Fig 1. Quantitative detection of SCH 58500-specific DNA sequences (mean ± SD) by real-time PCR analysis of samples from tumor (A) and nontumor bladder tissue (B) of untreated bladder cancer patients (control) and patients treated with intravesical instillation of SCH 58500 at dose levels 1 (B1), 2 (B2), and 3 (B3).

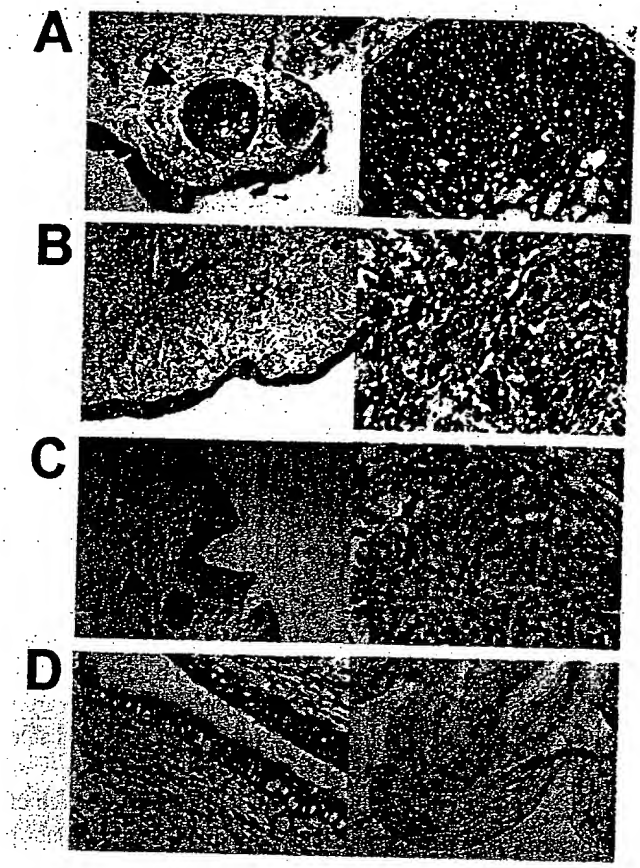

Fig 2. SCH 58500 vector distribution (in situ PCR) in tissue sections from patient no. 011 (A and B) and patient no. 005 (C) treated with intravesical instillation. Arrowheads indicate submucosal tumor nodules (A and C); arrow indicates cells in the Lamina propria (B). Sections from rat bladders injected with SCH 58500 (D, left panel) or control virus (D, right panel) are shown as positive and negative controls.

in submucosal tumor nodules as well as in cells in the Lamina propria. Thus, intravesical instillation of SCH 58500 in combination with a transduction-enhancing agent can achieve an uniform vector penetration throughout the urothelium as well as into submucosal tumor tissues.

Virologic Studies

After SCH 58500 treatment, all patients in both study groups underwent extensive bladder irrigation with 6 L saline through a transurethral catheter over a period of 36 to 48 hours. Excretion of infectious adenoviruses was detected by a sensitive flow cytometry-based assay[30] in samples taken from the first 2 to 4 L of void volume. No detectable urinary adenovirus excretion was found after 6 L of bladder irrigation (Fig 3). None of the urine samples taken 24 hours after study treatment gave a positive result in the qualitative on-site ELISA assay (not shown).

Long-Term Follow Up

Nine of the 12 study patients were alive at a median follow-up of 30 months. In addition to SCH 58500 treatment and radical cystectomy, two patients received a platinum-based adjuvant chemotherapy regimen. In one patient treated in part A, fulminant liver metastases developed 4 weeks after surgery, which were not detectable on computed tomogram and ultrasound examinations performed at the preoperative staging. The patient was treated with palliative chemotherapy, but he died from progressive liver failure 7 weeks after cystectomy. One patient treated in part A developed a *Mycoplasma* pneumonia during adjuvant chemotherapy. In total, three patients died from disease progression, and one patient is being treated with palliative chemotherapy for recurrent disease.

DISCUSSION

A major challenge in the conservative management of localized bladder cancer is the frequent recurrence and progression to an advanced tumor stage in patients with high-risk tumors.[2] To improve disease control, local tumor

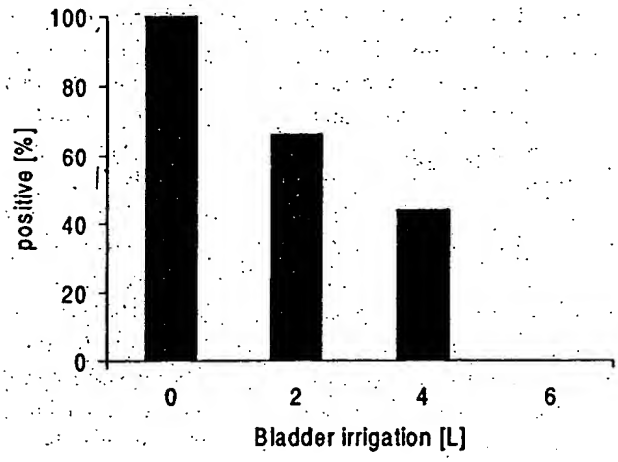

Fig 3. Excretion of infectious adenoviruses after intravesical SCH 58500 treatment. The percentage of patients (n = 12) with urine samples positive for infectious adenoviruses after the indicated volumes of bladder irrigation with saline.

resection is combined with intravesical therapy with bacillus Calmette-Guerin or anticancer agents.[8] However, these treatments have a substantial toxicity and may reduce the risk of recurrences but do not prevent disease progression.[6,7] Thus, new treatment options for high-risk superficial bladder cancer are required. Mutations of the *p53* tumor suppressor gene are frequently found in bladder cancer, are associated with an adverse prognosis in some studies, and may contribute to a more aggressive clinical course and resistance to anticancer treatment.[14,15] In an ortopic injection model of bladder cancer, *p53* gene transfer acted synergistically with cisplatin to prevent tumor growth and induce apoptosis in vivo.[41]

In the present phase I study, we tested whether adenoviral vector-mediated wild-type *p53* gene transfer is safe, feasible, and biologically active in patients with invasive bladder cancer. Taking advantage of the anatomy of the bladder, we planned to evaluate two different modes of vector administration: the intratumoral injection of vector solution, because it has been performed in several clinical studies of cancer gene therapy,[19-21] and the intravesical vector instillation via a transurethral catheter. Because preclinical studies convincingly demonstrated that the transduction efficacy of adenoviral vectors instilled into the bladder can be dramatically enhanced by the addition of several compounds,[27,28] here we administered intravesical SCH 58500 in combination with the transduction-enhancing agent Big CHAP.[28] Both modes of administration of the study treatment, intratumoral injection at cystoscopy and transurethral intravesical instillation, were well tolerated and devoid of any detectable systemic toxicity. Successful gene transfer after intravesical instillation of SCH 58500 in combination with Big CHAP was detected by RT-PCR analysis in seven of eight assessable patients. Moreover, evidence for biologic activity of the transgene, as determined by quantitative RT-PCR analysis of RNA expression as well as by immunohistochemical analysis of protein expression of the p53 target gene *p21/WAF1*,[32] was found in patients treated at higher dose levels. Transgene expression did not seem to correlate with the CAR expression status of the tumor samples as determined by RT-PCR analysis and immunohistochemistry. However, the relatively small number of patients enrolled onto this study and the detection methods for CAR expression might have influenced this result. Compared with the effective transduction achieved by intravesical vector instillation, no evidence for transgene expression was detected in the three patients treated by intratumoral injection of SCH 58500 at dose level 1, whereas SCH 58500 DNA sequences were detectable in two patients by PCR analysis. This was surprising, given that in a previous study in patients with NSCLC treated by intratumoral injection of SCH 58500, *p53* transgene expression was detected in four of five assessable patients receiving the same vector dose of 7.5×10^{11} particles.[20] Because intratumoral vector injection at cystoscopy is a relatively invasive procedure compared with transurethral vector instillation, it was decided not to proceed with the dose escalation in part A of this trial. Hence, we cannot exclude that at higher dose levels, a *p53* transgene expression in bladder tumors would have been achieved by intratumoral injection of SCH 58500 at cystoscopy. Furthermore, the addition of Big CHAP or other transduction-enhancing agents[28] might also be beneficial in the case of intratumoral vector injection in the bladder. However, in the light of the efficacy and ease of the intravesical instillation approach, intratumoral vector injection at cystoscopy clearly is the inferior approach for vector administration in bladder cancer.

In contrast to the results obtained with the p53 target gene *p21/WAF1*, we found no consistent changes in the expression of *p53*, various cell cycle–related or apoptosis-related genes, or TUNEL staining in response to SCH 58500 administration. This observation might be limited by the small number of patients enrolled onto the trial and the availability of only a single time point for these examinations. Moreover, the activity of many genes regulating apoptosis is not controlled by their expression level but by conformational changes or changes in their subcellular localization,[42] which cannot be detected by the methods applied in this study. Nevertheless, the *p21/WAF1* response is a valid marker for biologic activity of transgenic p53,

which has been confirmed in additional settings of clinical p53 gene therapy.[35,43]

In addition to molecular and immunohistochemical evidence for transgene expression and biologic activity, important information related to the vector distribution throughout the bladder and vector penetration into tumor tissues was gathered from this trial. We demonstrated by quantitative PCR analysis that administration of higher particle doses resulted in the recovery of higher copy numbers of SCH 58500-specific DNA from tissue samples (Fig 3). This was not unexpected; however, it suggests that together with the evidence for increased *p21/WAF1* expression at high doses, a plateau of the biologic activity was not reached by the intravesical instillation of 7.5×10^{13} particles SCH 58500. Presently, technical limitations preclude the administration of a more concentrated adenovirus solution, leaving this issue unresolved. With respect to the vector distribution after intravesical instillation, we found a uniform distribution of SCH 58500 DNA throughout the normal urothelium and the luminal tumor tissues by in situ PCR analysis of bladder sections. Moreover, vector DNA could also be found in apparently submucosal tumor nodules as well as in cells in the Lamina propria. These results confirm the hypothesis that the instillation approach results in an improved vector distribution. In addition, they demonstrate that even submucosal tumor cells can be targeted by the luminal administration of an adenovirus in combination with a transduction-enhancing agent in the bladder.

The optimal dosing schedule for intravesical SCH 58500 instillation remains to be established. Because of the procedure-associated discomfort observed in most patients treated in part B of this study, the contact times varied considerably. Yet SCH 58500 penetration and transgene expression analyses yielded promising results. It seems likely that even shorter contact times than the ones allowed in the course of this trial might result in sufficient transduction rates with lower local toxicity, a hypothesis supported by initial data from preclinical in vivo models. The intravesical instillation of SCH 58500 through a transurethral catheter also is environmentally safe, because infectious adenoviruses excreted in the urine after SCH 58500 treatment can easily be recovered in a contained system. The virologic studies performed in this trial suggest that if the bladder is sufficiently irrigated, infectious viral particles are only excreted with the first 4 L of irrigation fluid. This could minimize hospitalizations and could even allow outpatient treatment.

The design of the present phase I study precluded the collection of data regarding the long-term effects of the intravesical administration of such high-vector doses as well as signs for clinical efficacy. However, important and unique data demonstrating effective vector distribution, transgene expression, and biologic activity after a clinically practicable and safe gene transfer procedure were obtained in patients with invasive bladder cancer. These results provide a strong rationale for future investigation of adenovirus therapy in bladder cancer and support trials addressing the clinical efficacy of intravesical SCH 58500 treatment in patients with superficial high-risk bladder cancer.

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Adenovirus-Mediated Wild-Type *p53* Gene Transfer in Patients Receiving Chemotherapy for Advanced Non–Small-Cell Lung Cancer: Results of a Multicenter Phase II Study

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Purpose: **To study the additional benefit from adenoviral *p53* gene therapy in patients undergoing first-line chemotherapy for advanced non–small-cell lung cancer (NSCLC).**

Patients and Methods: **Twenty-five patients with nonresectable NSCLC were enrolled in an open-label, multicenter phase II study of three cycles of regimen A, carboplatin (area under the curve, 6; day 1) plus paclitaxel (175 mg/m^2, day 1), or regimen B, cisplatin (100 mg/m^2, day 1) plus vinorelbine (25 mg/m^2, days 1, 8, 15, and 22) in combination with intratumoral injection of 7.5 × 10^{12} particles of SCH 58500 (rAd/*p53*, day 1). Responses of individual tumor lesions were assessed after each cycle, and gene transfer was examined in posttreatment tumor biopsies using reverse transcriptase polymerase chain reaction.**

Results: **There was no difference between the response rate of lesions treated with *p53* gene therapy in addition to chemotherapy (52% objective responses) and lesions treated with chemotherapy alone (48% objective responses). Subgroup analysis according to the chemotherapy regimens revealed evidence for increased mean local tumor regressions in response to additional *p53* gene therapy in patients receiving regimen B, but not in patients receiving regimen A. There was no survival difference between the two chemotherapy regimens, and the median survival of the cohort was 10.5 months (1-year survival, 44%). Transgene expression was confirmed in tumor samples from 68% of patients, and toxicities attributable to gene therapy were mild to moderate.**

Conclusion: **Intratumoral adenoviral *p53* gene therapy appears to provide no additional benefit in patients receiving an effective first-line chemotherapy for advanced NSCLC.**

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LUNG CANCER IS a leading cause of death from malignancies in the Western World, and non–small-cell lung cancer (NSCLC) accounts for 75% to 80% of all lung cancers.[1] Usually, the only curative treatment available is surgical resection, an option limited to patients diagnosed in early stages of the disease and without significant comorbidity precluding thoracotomy. Patients with nonresectable tumors are treated with palliative chemotherapy or radiotherapy, but the expected survival of patients with stages III B and IV is still less than 20% at 2 years. Thus, new treatments for patients with advanced NSCLC are clearly required.

Structural alterations of the p53 tumor suppressor protein are observed in approximately 50% of tumors of NSCLC patients,[2] and in some, but not all, studies *p53* mutations are associated with an adverse prognosis.[3-5] Mutations of *p53* result in an impaired cellular response to various stresses, including DNA damage, growth factor withdrawal, and oncogenic transformation as well as to genomic instability.[6] Moreover, *p53* loss may also abrogate an effective apoptotic response to chemotherapy or radiation treatment.[7] In preclinical tumor models, *p53* gene transfer as mediated by retroviral or adenoviral expression vectors restored drug and radiation sensitivity or directly induced apoptosis.[8-15] On the basis of its preclinical efficacy, adenovirus-mediated wild-type (wt) *p53* gene transfer[16,17] or wt *p53* gene transfer in combination with cisplatin chemotherapy[18] was studied in phase I trials in patients with advanced NSCLC. These studies established the safety and feasibility of injections of adenoviral *p53* expression vectors into tumor lesions from patients with NSCLC. Moreover, they provided evidence for in vivo transgene expression,[16,17] as well as for efficacy

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in terms of surrogate markers of biologic activity[17,18] and some tumor remissions.[18]

The current multicenter phase II study of local adenovirus-mediated *p53* gene therapy was designed to systematically assess the clinical efficacy of this novel therapeutic approach in patients with incurable NSCLC undergoing an effective first-line chemotherapy.

PATIENTS AND METHODS

Patients

Adult patients with histologically confirmed, chemotherapy-naive, incurable NSCLC with a life expectancy of at least 4 months, a Karnofsky performance score of at least 70%, and absence of any clinical and laboratory evidence for dysfunction of hematopoietic, liver, renal, or coagulation systems were immunohistochemically or molecularly (sequencing or single-strand conformation polymorphism analysis) screened for intratumoral mutation or deletion of the *p53* gene as previously described.[16] Only patients with mutated or deleted *p53* and with two measurable tumor lesions in the same organ that were reasonably comparable in size were eligible for enrollment. An interval of at least 4 weeks between prior surgery and study treatment was mandatory, and all patients were required to provide written informed consent. Pregnant or nursing patients, fertile women not practicing medically accepted contraception, patients with acute adenoviral infections, immunosuppressed patients, HIV-positive patients, patients with uncontrolled serious infections, and patients with a history of acute or chronic respiratory distress were not eligible for enrollment.

Study Design

This was an international, multicenter, open-label, nonrandomized phase II trial. The protocol was approved by the local ethics committees and by the national regulatory offices of each participating center. The study was conducted according to the Declaration of Helsinki (amended version, Hong Kong, 1989), and following the principles of good clinical practice.

Treatment On day 1 of each cycle all patients received a single intratumoral injection of 10 mL of an aqueous solution containing a dose of 7.5×10^{12} particles SCH 58500 (rAd/*p53*), as described previously,[9,16,19] followed or preceded within 2 hours by the initiation of one of the two following chemotherapy regimens: (A) carboplatin (targeted area under the curve of 6, day 1) and paclitaxel (175 mg/m^2 during 3 hours, day 1), or (B) cisplatin (100 mg/m^2, day 1) and vinorelbine (25 mg/m^2, days 1, 8, 15, and 22). Treatment was repeated on day 22 for regimen A, and on day 29 for regimen B, and a maximum of three cycles was planned. Chemotherapy dose modifications were mandatory in case of hematopoietic toxicity. Chemotherapy and premedications were administered as per institutional practices. Intratumoral administration of SCH 58500 was performed either by percutaneous injection under computed tomographic (CT) guidance[20] or by intratumoral injection at bronchoscopy as a single-bolus injection central to the tumor lesion. The vector dose of 7.5×10^{12} particles was chosen based on its established safety as well as its efficacy to induce in vivo transgene expression in patients with advanced NSCLC.[16] After gene therapy, all patients were hospitalized in single rooms in a biosafety environment for at least 24 hours, or until adenovirus shedding was no longer detectable.

Study End Points and Evaluation Procedures The primary objective of the study was to compare the response rates of the SCH 58500-injected lesions to the response rates of the noninjected comparator lesions in patients receiving one of the two chemotherapy regimens. This design was chosen to permit detection of any additional local effect of intratumoral wt *p53* gene transfer by an intrapatient comparison. For response evaluation, CT scans of the chest and tumor measurements of all other extrathoracic tumor lesions were performed at screening, at the last day of each cycle, and every 2 months thereafter, if at least stable disease was achieved after the third cycle. To quantify changes in the size of individual lesions, the largest diameter of each lesion was multiplied with its perpendicular diameter after each treatment cycle, and was compared with the measurements taken at baseline. Remissions were classified according to National Cancer Institute criteria as complete remissions (CR), partial (decrease in the product of the largest and its perpendicular diameter by at least 50%) remissions (PR), stable disease (SD), or progressive (increase in size by at least 25%) disease (PD).

A secondary objective of the study was to assess the safety of the combination of the chemotherapy regimens with intratumoral injection of SCH 58500 for three cycles. Patients were closely monitored for any adverse event after treatment, and after hospital discharge patients were evaluated on a weekly basis by outpatient visits at the study centers. The daily monitoring during the first three posttreatment days included assessment of clinical symptoms, a physical examination, measurements of weight, height, and performance status, recording of adverse events, and frequent assessments of vital signs. Detection of adenovirus shedding, serum chemistry, and urinalysis were performed before and on day 1 of each cycle. Hematology, coagulation profile, ECG, and chest radiograms were assessed before each cycle. Another secondary end point was to assess biologic activity of SCH 58500, as defined by reverse transcription and polymerase chain reaction (RT-PCR) detection of vector-specific wt *p53* RNA sequences in posttreatment tumor biopsies as previously described.[16] For this purpose, on day 2 of each cycle (approximately 24 hours after injection), needle biopsies of the SCH 58500-injected tumor lesions were obtained by the same route by which SCH 58500 had been administered.

Virology Studies Adenovirus shedding was monitored in sputum, stool and rectal swabs, and urine by means of a qualitative enzyme-linked immunosorbent assay (ELISA) before treatment and daily until hospital discharge of the patient, as previously described.[16] Anti-adenovirus type 5 (Ad5) antibodies were detected using a previously described ELISA technique.[16] Neutralizing anti-adenoviral antibodies were assessed by means of the Saos-2 cell proliferation assay. In addition, urine and stool samples were assayed for the presence of infectious adenoviruses by a previously described method[21]: In brief, urine or stool swabs were diluted in growth medium and filter-sterilized. Then, 293 cells growing in logarithmic phase were incubated with the medium and sample mixes for 48 hours, and cells were harvested, stained with a fluorescent-labeled anti-Ad5 antibody, and analyzed by flow cytometry. Infectious titers were expressed as units per milliliter from the number of positive fluorescent cells measured. Any samples scored positive were reincubated with 293 cells for 48 hours. Cells were harvested, DNA was extracted for polymerase chain reaction (PCR) analysis according to standard techniques, and the presence of SCH 58500 sequences was tested using vector-specific primers.[16]

RESULTS

Enrollment and Treatments

Twenty-five patients from eight centers were enrolled in the study between September 24, 1997, and August 7, 1998.

Table 1. Patient Demographics and Localization of Tumor Lesions Receiving SCH 58500 Injections (SCH 58500-treated) and Lesions Serving as Intrapatient Control (comparator lesion)

Patient No.	Age (years)	Sex	Histology	Tumor Stage	SCH 58500-Treated	Comparator Lesion
Carboplatin and paclitaxel treatment						
001	60	male	adenocarcinoma	III B	tumor LLL	tumor tracheobronchial
002	35	female	large cell	IV	tumor RUL	tumor R hilar
004	57	male	squamous	IV	liver met. seg. 5	liver met. seg. 4
006	56	male	squamous	III B	tumor mediastinal	tumor L lung
007	67	male	adenocarcinoma	IV	tumor RUL	tumor R hilar
008	66	male	squamous	III B	ln. met. R supraclavicular	ln. met. tracheobronchial
011	62	male	undifferentiated NSCLC	IV	liver met. seg. 6	liver met. seg. 8
013	51	female	squamous	IV	tumor RUL	tumor LUL
014	83	male	undifferentiated NSCLC	III B	ln. met. supraclavicular	ln. met. aortopulmonal
016	80	female	squamous	IV	tumor RUL	tumor R pleural
017	77	male	undifferentiated NSCLC	III A	tumor L hilar	ln. met. paraaortal
019	44	female	adenocarcinoma	IV	ln. met. L suprascapular	ln. met. L supraclavicular
022	68	male	squamous	III B	ln. met. subcarinal	ln. met. pretracheal
Cisplatin and vinorelbine treatment						
003	50	male	large cell	IV	tumor RML	tumor LUL
005	48	male	adenocarcinoma	III B	tumor LLL	second tumor LLL
009	61	male	squamous	IV	tumor LUL	ln. met. R central
010	56	male	squamous	IV	tumor RUL	tumor lingula L
012	70	male	adenocarcinoma	IV	tumor RUL	ln. met. paratracheal
015	60	male	squamous	IV	chest wall mass	tumor R lung
018	65	female	adenocarcinoma	IV	tumor LLL	tumor RUL
020	50	male	squamous	IV	tumor RML	ln. met. subcarinal
021	47	male	squamous	IV	tumor LUL	ln. met. central
023	57	male	adenocarcinoma	III A	tumor RUL	ln. met. pretracheal
024	56	female	squamous	II B	tumor LUL	second tumor LUL
025	51	male	adenocarcinoma	II B	ln. met. pretracheal	ln. met. mediastinal

Abbreviations: L, left; R, right; LUL, left upper lobe; LLL, left lower lobe; RUL, right upper lobe; RML, right middle lobe; RLL, right lower lobe; ln. met. lymph node metastasis; seg, segment.

Patient characteristics and location of the tumor lesions assessed within the study are summarized in Table 1. A total of 68 treatment cycles were administered in 25 patients, with 20 patients completing the planned three cycles (one patient received two additional cycles, and two patients received one additional cycle each). Three patients received two treatment cycles, and two patients received only one cycle each. In 20 patients, SCH 58500 was administered by percutaneous intratumoral injection under CT guidance, whereas five patients received SCH 58500 by intratumoral injection at bronchoscopy. Thirteen patients were treated with a total of 37 (plus additional four) cycles of chemotherapy regimen A (carboplatin and paclitaxel). Twelve patients received a total of 31 cycles of chemotherapy regimen B (cisplatin and vinorelbine). The two treatment groups were balanced with respect to age and tumor stages (Table 1). Two patients treated with regimen A received intratumoral injections of SCH 58500 into liver metastases. All other patients received the vector injections into thoracic tumors or thoracic lymph node metastases (Table 1). Quantitative tumor responses were assessable for 66 treatment cycles. Tumor measurements were missing for one cycle each in two patients treated with regimen A, and in one patient treated with regimen B.

Clinical Responses

To detect a possible local benefit from additional wt *p53* gene therapy in combination with chemotherapy, the study was designed to analyze for each individual patient the variation of the local responses of two tumor lesions comparable in size and located within the same organ. When the isolated responses of the SCH 58500-injected lesions and the comparator lesions after each patient's last cycle of study therapy were assessed, 52% of the lesions injected with SCH 58500 and 48% of the comparator lesions had an objective response (Table 2). Similarly, no differences were observed when the patients were analyzed separately according to their chemotherapy regimen (54% *v* 46% responses for carboplatin and paclitaxel, and 50% *v* 50% for cisplatin and vinorelbine). In six patients (one treated with regimen A and five treated with regimen B), SCH 58500 was injected into primary lung tumors, and

Table 2. Local Tumor Responses of Lesions*

Patient No.	Cycles	SCH 58500-Treated	Comparator Lesion	Neutral. Ab	RT-PCR
Carboplatin and paclitaxel treatment					
001	3	PR	PR	+	+
002	1	StD	StD	+	+
004	3	PD	PD	+	+
006	3	PR	StD	+	+
007	2	StD	StD	+	–
008	3	PR	PR	+	+
011	3	StD	PR	+	–
013	3	StD	StD	+	+
014	3	CR	PR	+	+
016	2	StD	PD	nd	+
017	3	PR	PR	+	–
019	3	PR	PR	+	+
022	3	PR	StD	+	+
Cisplatin and vinorelbine treatment					
003	3	StD	CR	+	+
005	3	StD	PD	NB	–
009	2	StD	PR	nd	–
010	3	PR	StD	+	–
012	3	StD	PR	+	–
015	3	PR	PR	nd	+
018	2	PR	PR	+	–
020	3	PR	PR	+	+
021	1	StD	StD	nd	+
023	3	CR	StD	NB	+
024	3	StD	StD	+	+
025	3	PR	StD	+	+

Abbreviations: CR, complete response; PR, partial response; StD, stable disease; PD, progressive disease; nd, not determined; NB, no baseline value available; Neutral. Ab, neutralizing anti-SCH 58500 antibodies; RT-PCR, reverse transcriptase polymerase chain reaction.

*Table represents responses of lesions treated with SCH 58500 injection in addition to systemic chemotherapy and of noninjected lesions comparable in size and located within the same organ (comparator lesion) after the last cycle each patient received within the trial, development of neutralizing anti-SCH 58500 antibodies and RT-PCR detection of transgene expression in posttreatment biopsies.

thoracic lymph node metastases served as comparator lesions (Table 1). No overall difference between the responses of primary tumors (one CR, two PR, and three SD) and lymph node metastases (three PR and three SD) was noted in these six patients (Table 2).

When the areas of the SCH 58500-treated lesions and the comparator lesions were calculated at the end of each treatment cycle, a significant difference ($P = .028$, Wilcoxon signed rank test) in tumor regressions between the SCH 58500-treated lesions and the comparator lesions of all study patients could only be found after the second cycle (Fig 1A). However, when patients were analyzed separately for each of the two chemotherapy regimens, a different picture emerged: In the 13 patients receiving carboplatin and paclitaxel (regimen A), there was no obvious difference between the mean response of the SCH 58500-treated and the comparator lesions after any of the three cycles (Fig 1B). After the third cycle, a mean regression of approximately 60% was observed in lesions receiving additional gene therapy and in the comparator lesions. In contrast, the mean regression of the comparator lesions of patients who where treated with cisplatin and vinorelbine (regimen B) was only 15%, whereas it amounted to 55% in lesions that were additionally injected with SCH 58500 (Fig 1C). Because of the small sample numbers of the subgroups, no statistical level of significance was calculated.

Regarding the overall outcome of the patients, there was no significant survival difference between the two chemotherapy regimens: Median survival was 10 months in 13 patients treated with carboplatin and paclitaxel, and 13.5 months in 12 patients treated with cisplatin and vinorelbine ($P = .328$, log-rank test). The median survival of the complete cohort was 10.5 months, and survival at 1 year was 44% (Fig 2).

Detection of p53 *Transgene Expression*

Intratumoral expression of vector-specific wt *p53* RNA could be detected in 12 of 25 patients (48%) undergoing posttreatment biopsies approximately 24 hours after their first SCH 58500 treatment. Another five of 11 patients who had negative RT-PCR results after the first cycle and who underwent additional biopsies had evidence for intratumoral transgene expression after subsequent treatment cycles. Thus, a total of 17 of 25 patients (68%) exhibited transgene expression by positive RT-PCR for vector-specific wt *p53* sequences (Table 2).

Toxicities

Toxicities attributable to treatment with SCH 58500 generally were mild to moderate. Most frequently fever, influenza-like symptoms, nausea or anorexia, and fatigue were observed. Injection-site complications occurred during only five of 53 cycles in which SCH 58500 was administered by CT-guided percutaneous injection (one with World Health Organization [WHO] grade 2 pain and four with WHO grade 1 reactions). All toxicities of WHO grade 2 or higher, which occurred during at least two of 68 cycles, are summarized in Table 3.

Unscheduled hospitalizations occurred because of decreased performance status (two patients), renal failure (one patient after an additional fourth cycle), febrile neutropenia (one patient), bacteriemia in the absence of fever (one patient), and hypercalcemia (one patient), none of which was considered as probably related to SCH 58500 treatment. In five patients the study had to be discontinued prematurely: three patients had disease progression after the second cycle, one patient experi-

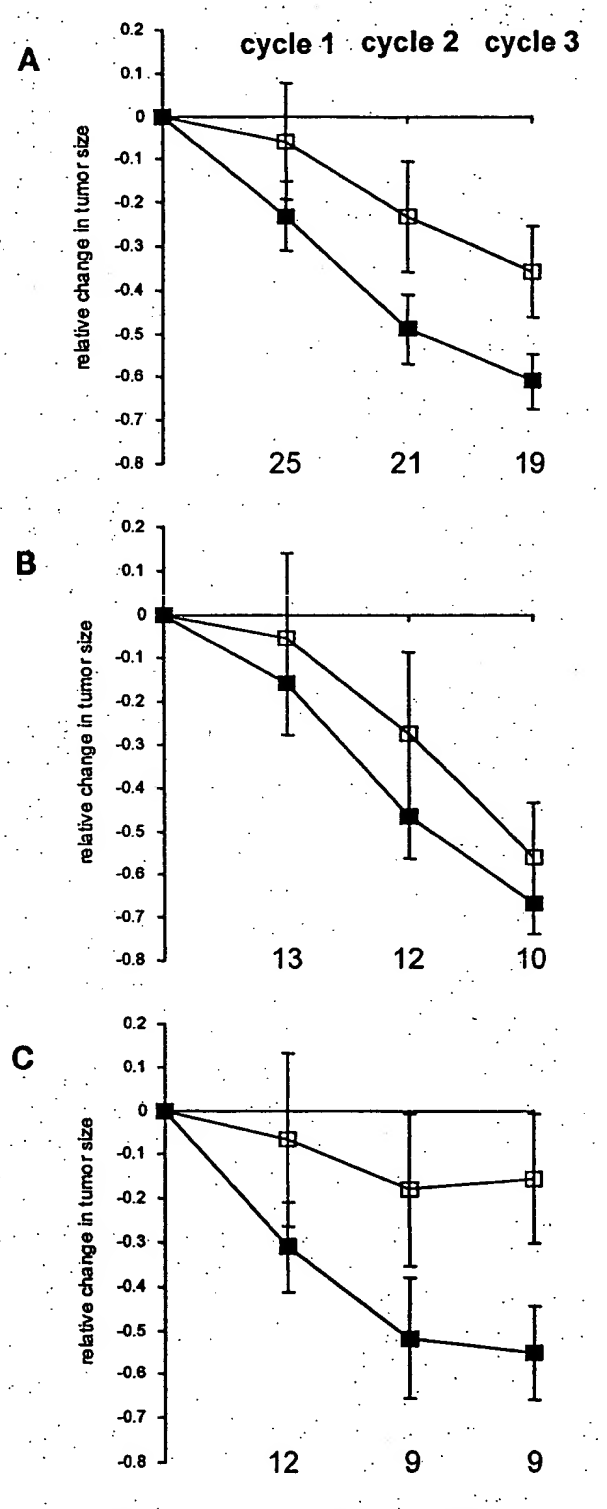

Fig 1. Quantitative tumor responses after wt *p53* gene therapy plus chemotherapy. Mean relative areas changes ± SE of SCH 58500-injected tumors (closed squares) and comparator lesions (open squares) of the whole study cohort (A), patients treated with carboplatin and paclitaxel (B), and patients treated with cisplatin and vinorelbine (C). (Numbers at bottom of each graph indicate the number of assessable patients.)

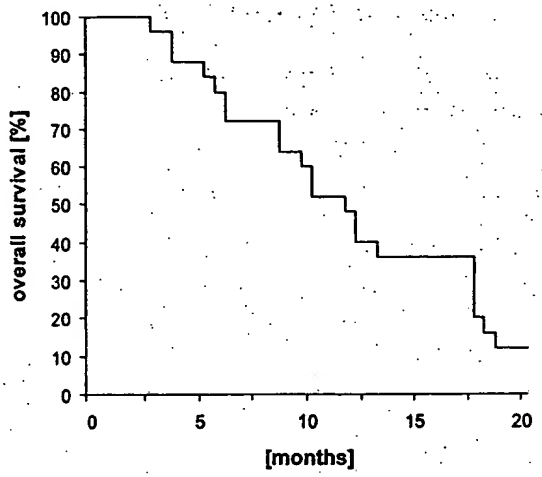

Fig 2. Actuarial survival of 25 patients treated with intratumoral injections of SCH 58500 in combination with chemotherapy (Kaplan-Meier analysis).

enced asthenia and leukocytopenia after the first cycle leading to discontinuation, and one patient had a remission of the injected lesion after the first cycle, which made it technically impossible to reinject SCH 58500 during subsequent cycles.

Changes in laboratory variables were mainly restricted to blood counts, which was not unexpected in the light of the known toxicities of the two chemotherapy regimens ap-

Table 3. Treatment-Related Toxicities of at Least WHO Grade 2 Observed in at Least Two of 68 Cycles of SCH 58500-Injection in 25 Patients

Toxicity	WHO Grade 2	3	4
Anorexia	9	—	—
Arthralgia	2	—	—
Asthenia	5	1	—
Constipation	6	—	—
Cough	4	1	—
Dehydration	3	—	—
Diarrhea	6	—	—
Dyspnea	3	2	—
Emesis	4	2	—
Fatigue	8	3	—
Fever	18	1	—
Headache	1	1	—
Hypertension	3	—	—
Influenza-like symptoms	2	1	—
Myalgia	2	—	—
Nausea	14	—	—
Pain	12	1	—
Sweating	2	—	—

NOTE. The numbers indicate the maximal severity of the respective adverse event per subject.

plied. A WHO grade of greater than 3 leuko- or neutropenia occurred in six of 13 patients (46%) treated with regimen A (carboplatin and paclitaxel), and in 11 of 12 patients (92%) treated with regimen B (cisplatin and vinorelbine). A WHO grade of greater than 3 thrombocytopenia was observed in two of 13 patients (15%) receiving regimen A, and one of 12 patients (8%) receiving regimen B. In addition, a WHO grade 2 increase of transaminases was observed in three patients, and a WHO grade 2 increase of alkaline phosphatase was observed in one patient treated with regimen A. Four patients treated with regimen B experienced a WHO grade 2 increase in transaminases. One patient treated with regimen A had a WHO grade 3 renal insufficiency after a fourth treatment cycle, which he received outside the protocol. In another patient, a reversible WHO grade 2 hyponatremia was observed.

Virology Studies

In two of 25 patients adenovirus shedding in the urine was detected by means of the on-site ELISA. Excretion of infectious adenoviral particles (4.35×10^4 U/mL) was detected in a stool sample from one patient (patient no. 011), obtained 24 hours after SCH 58500 injection during the second cycle. However, no vector-specific DNA sequences were found in this sample using PCR. No adenoviruses were detected in a urine sample taken at the same time, or in stool and urine samples obtained 48 hours after treatment. This patient was one of the two patients receiving SCH 58500 injections into liver metastases (Table 1).

Positive anti-Ad5 antibody titers were detected in all 19 patients for whom pretreatment serum samples were available. After the first injection of SCH 58500, the anti-Ad5 serum antibody titers increased by a mean of 3.5-fold at day 7, and by a mean of 35-fold at day 14 after injection (Fig 3A). During subsequent treatment cycles anti-Ad5 antibody titers remained at these increased levels (data not shown). In none of the 19 patients in whom pretreatment serum analysis could be performed were neutralizing anti-SCH 58500 serum antibodies detectable at baseline. After SCH 58500 injection, neutralizing antibodies developed in all of these 19 patients. Neutralizing antibodies were detectable as early as day 7 of the first cycle (Fig 3B) and remained detectable in all patients assessed throughout the study (data not shown).

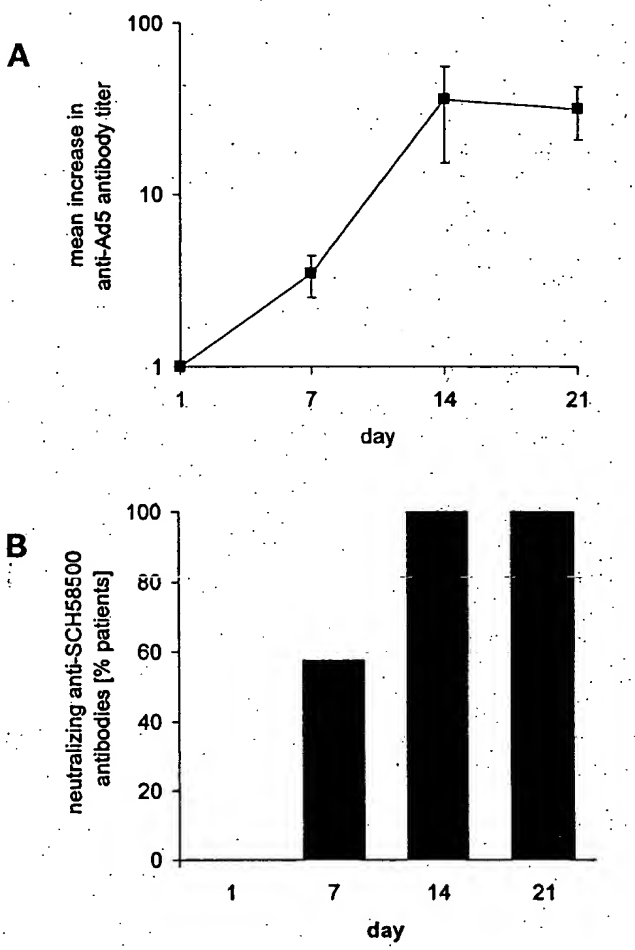

Fig 3. Development of anti-adenoviral serum antibodies and neutralizing anti-SCH 58500 serum antibodies after the first treatment with SCH 58500 in 19 assessable patients. (A) Mean increase (± SE) above baseline of anti-adenoviral antibodies. (B) Fraction of patients with detectable neutralizing anti-SCH 58500 antibodies.

DISCUSSION

Transfer of the tumor suppressor gene *p53* was shown to induce permanent cell cycle arrest or apoptotic cell death in a large number of cancer cell lines, as well as in several preclinical animal models of cancer.[10,13,22,23] An apoptotic response to *p53* activation appears to be limited to transformed cells, whereas nontransformed cells mainly undergo cell cycle arrest after *p53* activation.[24,25] Encouraging preclinical results led to a number of early clinical studies exploring vector-mediated *p53* gene transfer in advanced cancer patients. The earlier studies were performed in patients with incurable NSCLC,[16,26] or head and neck squamous cell cancer.[27] A pilot study[26] in which retroviral *p53* expression vectors were directly injected into small endobronchial lesions received widespread publicity and gave increase to high hopes for the efficacy of *p53* gene therapy of cancer. As the vector technology applied in these trials did not allow selective tumor targeting, and systemic administration of these vectors was precluded because of their high immunogenicity and the high prevalence of cross-reacting antibodies, direct approaches of tumor target-

ing were applied. The safety and feasibility of intratumoral injection of adenoviral wt *p53* expression vectors was established in NSCLC patients[16,17] and in patients with head and neck squamous cell cancer.[27] Furthermore, evidence for transgene expression, and possibly induction of apoptosis, was documented by these trials.[16,17,27] Additional trials evaluated infusion of adenoviral vectors into body cavities[28] or tumor perfusion through intra-arterial injection of vector solutions[29] as other targeting techniques.

Intratumoral vector injection can only be applied to a limited number of lesions per patient, and currently there is no clinical evidence to support systemic antitumoral effects from such a treatment. Thus, it is difficult to assess the clinical activity of local *p53* gene transfer in patients with advanced NSCLC after standard oncologic practice. Furthermore, response data from controlled phase I studies of adenoviral wt *p53* gene transfer[16,17] did not reproduce the impressive local effects, which were initially reported using a less-effective retroviral expression system.[26] To overcome those limitations, the novel design of the present study was chosen. By comparing the isolated responses of a tumor lesion treated with adenoviral wt *p53* gene transfer with a comparable lesion not receiving gene therapy in patients undergoing first-line chemotherapy, we hoped to obtain a meaningful assessment of additional effects from local wt *p53* gene therapy in NSCLC. These restrictive inclusion criteria naturally raise the risk of selection bias in our study. However, we believe that our observations are valid, inasmuch as responses were not compared with historical controls but within each individual patient, thus minimizing the influence of biologic differences among patients as well as their tumors on the efficacy outcome.

In doing so, we found no convincing evidence for an additional local benefit from adenoviral wt *p53* gene transfer in NSCLC patients undergoing first-line chemotherapy. This result also held true, if quantitative regressions of mean tumor sizes were compared instead of the overall responses of each lesion (Fig 1A). One possible explanation for this observation could be the relatively high efficacy of the chemotherapy administered, as well as the small cohort size of our study. Response rates of 50% certainly are in the upper range of those reported in phase II studies of chemotherapy alone.[30-32] However, this is not surprising given the relatively good performance status of the patients included in this study.

Second, the lack of a detectable clinical benefit from SCH 58500 injection could hypothetically result from biologic inactivity of the study medication in our study patients. This, however, appears highly unlikely, as this vector formulation has extensively demonstrated biologic activity in various preclinical tumor models comparable to the clinical setting of this trial.[11,33,34] Moreover, transgene expression as determined by RT-PCR analysis in 24-hour posttreatment tumor biopsies was confirmed in 68% of the study patients (Table 2). As this trial was mainly designed to address the clinical efficacy end point, no systematic studies of additional surrogate markers for treatment-associated induction of cell cycle arrest or apoptosis were performed. However, using quantitative RT-PCR methodology, upregulation of mRNA of the *p53*-target gene *p21/WAF1* after SCH 58500 administration was observed in a subgroup of this study cohort.[35]

Another reason for the lack of an additional benefit from *p53* gene therapy might be insufficient spreading of the replication-defective adenoviral vectors within the tumors after central intralesional injection. As surgery was not indicated in the patients enrolled in the present trial, this factor could not be assessed in this study. Addressing this important question should be considered in future studies of adenoviral cancer gene therapy performed in early stage patients scheduled for surgery. Recently, the problem of ineffective vector spreading has been attempted to be overcome by administration of replication-competent adenoviruses,[36,37] and encouraging clinical results were reported.[38,39] However, using replication-competent viruses, even on the background of preferential replication in cells with mutations in the ARF-p53-Rb pathway, might pose additional safety concerns.

Toxicities observed in this study mainly resulted from the concomitant chemotherapy, and they were within the range of those described from large phase II or phase III trials of the same agents alone in advanced NSCLC patients.[30-32,40,41] Toxicities attributed to the gene therapy itself were mild to moderate, and confirmed results from phase I trials of adenoviral *p53* vector injection alone.[16,17]

Vector-specific wt *p53* RNA expression could be detected in posttreatment biopsies from a high number of patients (68%). This demonstrates a high quality of study conduct, which was important because the majority of centers enrolled only three or fewer patients in this multicenter trial. Although excretion of infectious adenoviral particles could be detected during only one of 68 treatment cycles, high titers of adenovirus-reacting antibodies and neutralizing anti-adenoviral antibodies became detectable in all assessable patients after gene therapy. These antibodies developed even though all patients received a highly myelotoxic chemotherapy and most patients were premedicated with dexamethasone. This supports the known immunogenicity of adenoviral vectors in humans. Median and 1-year survival, as well as the toxicities observed in this trial, compared favorably with those from studies of similar chemotherapy regimens,[30-32,40,41] establishing the safety of

multiple intratumoral injections of SCH 58500 in combination with chemotherapy in patients with advanced NSCLC.

Although our study patients were not randomly assigned to chemotherapy regimen A or B, retrospective subgroup analysis still provided some potentially interesting information. If the comparison of the chemotherapy response was limited to the comparator lesions, which did not receive additional gene therapy, higher mean tumor regressions were observed after treatment with carboplatin plus paclitaxel than after cisplatin plus vinorelbine (Fig 1, B and C). In the former group no additional benefit could be found in lesions treated with *p53* gene therapy. In contrast, the latter group exhibited a difference in the local effect from gene therapy combined with chemotherapy, which was most pronounced after the second and third cycles. This has to be interpreted with caution, given the small sample size and the retrospective nature of the analysis. However, this might suggest some additional local effects from adenovirus-mediated wt *p53* gene transfer on the background of a less than optimal response to systemic chemotherapy. In contrast, local *p53* gene therapy does not appear to provide an additional benefit, if the overall response to systemic chemotherapy is optimal.

Relevant benefits from adenovirus-mediated wt *p53* gene therapy to the large population of patients with advanced, inoperable NSCLC in terms of overall response rate and survival, however, can only be assessed in randomized phase III trials. Whether such a study is warranted on the basis of the results obtained in this first phase II study, using current vector technology, which is limited to local application, remains open.

ACKNOWLEDGMENT

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Development and Characterization of Recombinant Adenoviruses Encoding Human p53 for Gene Therapy of Cancer

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ABSTRACT

We have constructed recombinant human adenoviruses that express wild-type human p53 under the control of either the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early gene promoter. Each construct replaces the Ad 5 E1a and E1b coding sequences necessary for viral replication with the p53 cDNA and MLP or CMV promoter. These p53/Ad recombinants are able to express p53 protein in a dose-dependent manner in infected human cancer cells. Tumor suppressor activity of the expressed p53 protein was assayed by several methods. [^{3}H]Thymidine incorporation assays showed that the recombinant adenoviruses were capable of inhibiting DNA synthesis in a p53-specific, dose-dependent fashion. *Ex vivo* treatment of Saos-2 tumor cells, followed by injection of the treated cells into nude mice, led to complete tumor suppression using the MLP/p53 recombinant. Following a single injection of CMV/p53 recombinant adenovirus into the peritumoral space surrounding an *in vivo* established tumor derived from a human small cell lung carcinoma cell line (NIH-H69), we were able to detect p53 mRNA in the tumors at 2 and 7 days post-injection. Continued treatment of established H69 tumors with MLP/p53 recombinant led to reduced tumor growth and increased survival time compared to control treated animals. These results indicate that recombinant adenoviruses expressing wild-type p53 may be useful vectors for gene therapy of human cancer.

OVERVIEW SUMMARY

Introduction of the p53 tumor suppressor gene into tumors bearing p53 mutations can inhibit cellular proliferation and tumorigenicity. Wills *et al.* describe replication-deficient recombinant adenoviruses directing expression of human p53 both *in vitro* and *in vivo*. They show that adenovirus-mediated expression of wild-type p53 in p53 altered tumors can suppress proliferation and inhibit tumorigenicity in *ex vivo* and *in vivo* cancer models.

INTRODUCTION

MUTATION OF THE P53 GENE is the most common genetic alteration in human cancers (Bartek *et al.*, 1991; Hollstein *et al.*, 1991). In its proposed role as a "guardian of the genome" (Lane, 1992), the p53 gene product functions as a transcriptional activator of other genes which inhibit cell cycle progression from G_1 to S phase in normal cells. Its levels rise and accumulate in response to DNA damage, leading either to G_1 arrest and repair, terminal differentiation, or, if too much damage has occurred, apoptosis (Kuerbitz *et al.*, 1992; Lane, 1992). Loss of wild-type p53 function is associated with the uncontrolled growth of many types of human cancers. The reexpression of normal p53 in p53-altered tumor cells has been demonstrated to suppress tumor growth (Chen *et al.*, 1990; Cheng *et al.* 1992; Takahashi *et al.*, 1992) or induce apoptosis (Yonish-Rouach *et al.*, 1991, Shaw *et al.*, 1992). Therefore, p53 functions as a tumor suppressor, restoring a nontumorigenic phenotype to tumor cells in which the endogenous p53 gene has been deleted or mutated.

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Recent work has shown that human adenoviruses can be used to deliver genes successfully into a variety of cells and tissues (Lemarchand *et al.*, 1992; Rosenfeld *et al.*, 1992; Rich *et al.*, 1993). Recombinant adenoviruses have several advantages over alternative gene delivery systems such as retrovirus (RV) or adeno-associated virus (AAV)-based vectors for the treatment of cancer. These include the ability to produce stable, high-titer virus capable of efficient infection and subsequent gene expression in target cells (for review, see Siegfried, 1993). Because of the advantages of an adenovirus-based delivery system over other systems for the potential gene therapy of cancer, we constructed recombinant adenoviruses encoding wild-type p53 under the control of the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) promoter. We have tested the ability of these constructs to suppress tumor growth both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines

Recombinant adenoviruses were grown and propagated in the human embryonal kidney cell line 293 (ATCC CRL 1573) maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). Saos-2 cells were maintained in Kaighn's media supplemented with 15% fetal calf serum. HeLa and Hep 3B cells were maintained in DME medium supplemented with 10% fetal calf serum. All other cell lines were grown in Kaighn's media supplemented with 10% fetal calf serum. Saos-2 cells were kindly provided by Dr. Eric Stanbridge. All other cell lines were obtained from ATCC.

Construction of recombinant adenoviruses

To construct the Ad5/p53 viruses, a 1.4-kb *Hind* III–*Sma* I fragment containing the full-length cDNA for p53 was isolated from pGEM1-p53-B-T (kindly supplied by Dr. Wen-Hwa Lee) and inserted into the multiple cloning site of the expression vector pSP72 (Promega) using standard cloning procedures (Sambrook *et al.*, 1989). The p53 insert was recovered from this vector following digestion with *Xho* I–*Bgl* II and gel electrophoresis. The p53 coding sequence was then inserted into either pNL3C or pNL3CMV adenovirus gene transfer vectors (kindly provided by Dr. Robert Schneider), which contain the Ad5 5' inverted terminal repeat and viral packaging signals and the E1a enhancer upstream of either the Ad2 major late promoter (MLP) or the human cytomegalovirus immediate early gene promoter (CMV), followed by the tripartite leader cDNA and Ad 5 sequence 3,325–5,525 bp in a pML2 background. These new constructs replace the E1 region (bp 360–3,325) of Ad5 with p53 driven by either the Ad2 MLP (A/M/53) or the human CMV promoter (A/C/53), both followed by the tripartite leader cDNA (see Fig. 1). The p53 inserts use the remaining downstream E1b polyadenylation site. Additional MLP- and CMV-driven p53 recombinants (A/M/N/53, A/C/N/53) were generated which had a further 705-nucleotide deletion of Ad 5 sequence to remove the protein IX (pIX) coding region. As a control, a recombinant adenovirus was generated from the parental pNL3C plasmid without a p53 insert (A/M). A second

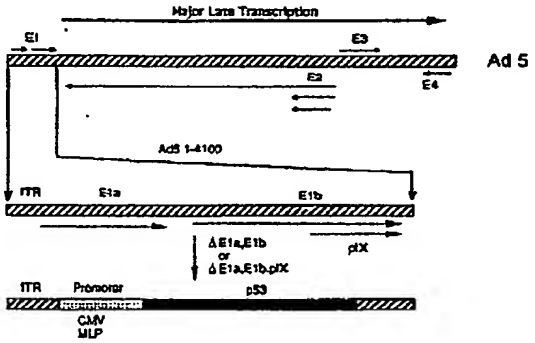

FIG. 1. Schematic of recombinant p53/adenovirus constructs. The p53 recombinants are based on Ad 5 and have had the E1 region of nucleotides 360–3,325 replaced with a 1.4-kb full-length p53 cDNA driven by the Ad 2 MLP (A/M/53) or human CMV (A/C/53) promoters followed by the Ad 2 tripartite leader cDNA. The control virus A/M has the same Ad 5 deletions as the A/M/53 virus, but lacks the 1.4-kb p53 cDNA insert. The remaining E1b sequence (705 nucleotides) have been deleted to create the protein IX-deleted constructs A/M/N/53 and A/C/N/53. These constructs also have a 1.9-kb *Xba* I deletion within adenovirus type 5 region E3.

control (kindly provided by Dr. Robert Schneider) consisted of a recombinant adenovirus encoding the β-galactosidase (β-Gal) gene under the control of the CMV promoter (A/C/β-Gal). The plasmids were linearized with either *Nru* I or *Eco* RI and co-transfected with the large fragment of a *Cla* I-digested Ad 5 *dl*309 or *dl*327 mutants (Jones and Shenk, 1979; Thimmappaya *et al.*, 1982) using a Ca/PO_4 transfection kit (Stratagene). Only the pIX-minus constructs used the *dl*327 background which contains a 1.9-kb *Xba* I deletion in the E3 region. Viral plaques were isolated and recombinants identified by both restriction digest analysis and the polymerase chain reaction (PCR) using recombinant-specific primers against the tripartite leader cDNA sequence with downstream p53 cDNA sequence. Recombinant virus was further purified by limiting dilution, and virus particles were purified and titered by standard methods (Graham and van der Erb, 1973; Graham and Prevec, 1991).

p53 protein detection

Saos-2 or Hep 3B cells (5×10^5) were infected with the indicated recombinant adenoviruses for a period of 24 hr at increasing multiplicities of infection (moi) of plaque-forming units of virus/cell. Purified adenovirus, stored in1% sucrose in phosphate-buffered saline (PBS), is diluted with media to obtain the desired moi and added to plates of cells containing fresh media. After 24 hr, the cells were washed once with PBS and harvested in lysis buffer [50 m*M* Tris-HCl pH 7.5, 250 m*M* NaCl, 0.1% NP-40, 50 m*M* NaF, 5 m*M* EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. A Bradford assay (Bio-Rad Protein Assay kit) was used to measure cellular protein concentration, and equal amounts of protein (approximately 30 μg) were separated

by 10% SDS-PAGE and transfered to nitrocellulose. Membranes were incubated with α-p53 antibody PAb 1801 (Novocastro) followed by sheep anti-mouse IgG conjugated with horseradish peroxidase. p53 protein was visualized by chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

Measurement of DNA synthesis rate

Cells (5×10^3/well) were plated in 96-well titer plates (Costar) and allowed to attach overnight (37°C, 7% CO_2). Cells were then infected for 24 hr with purified recombinant virus particles at moi values ranging from 0.3 to 100, as indicated. Media were changed 24 hr after infection, and incubation was continued for a total of 72 hr. [^{3}H]Thymidine (Amersham, 1 μCi/well) was added 18 hr prior to harvest. Cells were harvested on glass fiber filters and levels of incorporated radioactivity were measured in a beta scintillation counter. [^{3}H]Thymidine incorporation was expressed as the mean % (±SD) of media control and plotted versus the moi.

Tumorigenicity in nude mice

Approximately 2.4×10^8 Saos-2 cells, plated in T225 flasks, were treated with suspension buffer (1% sucrose in PBS) containing either A/M/N/53- or A/M-purified virus at an moi of 3 or 30. Following an overnight infection, cells were injected subcutaneously into the left and right flanks of BALB/c athymic nude mice (4 mice per group). One flank was injected with the A/M/N/53-treated cells, while the contralateral flank was injected with the control A/M-treated cells, each mouse serving as its own control. Animals receiving bilateral injection of buffer-treated cells served as additional controls. Tumor dimensions (length, width, and height) and body weights were then measured twice per week over an 8-week period. Tumor volumes were estimated for each animal, assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

Intratumoral RNA analysis

Female BALB/c athymic nude mice (approximately 5 weeks of age) were injected subcutaneously with 1×10^7 H69 small cell lung carcinoma (SCLC) cells in a 200-μl volume in their right flanks. Tumors were then allowed to progress for 32 days. Mice then received peritumoral injections of either A/C/53 or A/C/β-Gal recombinant adenovirus [2×10^9 plaque-forming units (pfu)] into the subcutaneous space beneath the tumor mass. Tumors were excised from the animals 2 and 7 days post adenovirus treatment and rinsed with PBS. Tumor samples were homogenized, and total RNA was isolated using a TriReagent kit (Molecular Research Center, Inc.). Poly(A) RNA was isolated using the PolyATract mRNA Isolation System (Promega), and approximately 10 ng of sample was used for reverse transcriptase (RT)-PCR determination of recombinant p53 mRNA expression (Wang *et al.*, 1989). Primers were designed to amplify sequence between the adenovirus tripartite leader cDNA and the downstream p53 cDNA, ensuring that only recombinant, and not endogenous p53 would be amplified.

p53 gene therapy of established tumors in nude mice

Approximately 1×10^7 H69 (SCLC) tumor cells in 200-μl volumes were injected subcutaneously into female BALB/c athymic nude mice. Tumors were allowed to develop for 2 weeks, at which point animals were randomized by tumor size (n = 5/group). Peritumoral injections of either A/M/N/53 or the control A/M adenovirus (2×10^9 pfu/injection) or buffer alone (1% sucrose in PBS) were administered twice per week for a total of 8 doses/animal per group. Tumor dimensions and body weights were measured twice per week for 7 weeks, and tumor volume was estimated as described previously. Animals were then followed to observe the effect of treatment on mouse survival.

RESULTS

Construction of recombinant p53-adenovirus

p53 adenoviruses were constructed by replacing a portion of the E1a and E1b region of adenovirus type 5 with p53 cDNA under the control of either the Ad2 MLP (A/M/53) or CMV (A/C/53) promoter (schematized in Fig. 1). This E1 substitution severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells that supply Ad 5 E1 gene products *in trans* (Graham *et al.*, 1977). After identification of p53 recombinant adenovirus by both restriction digest and PCR analysis, the entire p53 cDNA sequence from one of the recombinant adenoviruses (A/M/53) was sequenced to verify that it was free of mutations. Following this, purified preparations of the p53 recombinants were used to infect HeLa cells to assay for the presence of phenotypically wild-type adenovirus. HeLa cells, which are nonpermissive for replication of E1-deleted adenovirus, were infected with $1–4 \times 10^9$ infectious units of recombinant adenovirus at an moi = 50, cultured for 3 weeks, and observed for the appearance of cytopathic effect (CPE). Using this assay, we were not able to detect recombinant adenovirus replication or wild-type contamination, readily evident by the CPE observed in control cells infected with wild-type adenovirus at a level of sensitivity of approximately 1 in 10^9.

p53 protein expression from recombinant adenovirus

To determine if our p53 recombinant adenoviruses expressed p53 protein, we infected tumor cell lines that do not express endogenous p53 protein. The human tumor cell lines Saos-2 (osteosarcoma) and Hep 3B (hepatocellular carcinoma), which contain mutations that result in no expression of p53 protein (Chen *et al.*, 1990; Hsu *et al.*, 1993), were infected for 24 hr with the p53 recombinant adenoviruses A/M/53 or A/C/53 at moi values ranging from 0.1 to 200 pfu/cell. Western analysis of lysates prepared from infected cells demonstrated a dose-dependent p53 protein expression in both cell types (Fig. 2). Both cell lines expressed higher levels of p53 protein following infection with A/C/53 than with A/M/53 (Fig. 2). No p53 protein was detected in noninfected cells. Cells infected with moi values of up to 200 of the control virus A/M also did not show detectable p53 protein (unpublished observation). SW 480 cell

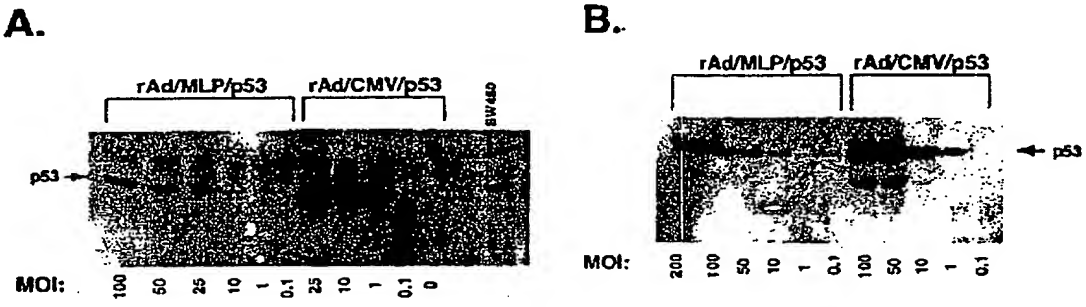

FIG. 2. p53 protein expression in tumor cells infected with A/M/53 and A/C/53. A. Saos-2 (osteosarcoma) cells were infected at the indicated moi with either the A/M/53- or A/C/53-purified virus and harvested 24 hr later. The p53 antibody pAb 1801 was used to stain immunoblots of samples loaded at equal total protein concentrations. Equal protein concentrations of SW480 cell extracts, which overexpress mutant p53 protein, were used as a marker for p53 size. The zero (0) under the A/C/53 heading indicates a mock infection containing untreated Saos-2 lysate. B. Hep 3B (hepatocellular carcinoma) cells were infected with the A/M/53 or A/C/53 virus at the indicated moi and analyzed as in A. The arrow indicates the position of the p53 protein.

lysate, which overexpresses mutant p53 protein (Baker *et al.*, 1990), was used as a size marker. Levels of endogenous wild-type p53 are normally quite low, and nearly undetectable by Western analysis of cell extracts (Bartek *et al.*, 1991). It is clear however that wild-type p53 protein levels are easily detectable after infection with either A/M/53 or A/C/53 at the lower moi values (Fig. 2), suggesting that even low doses of p53 recombinant adenoviruses can produce potentially efficacious levels of p53.

p53-dependent morphology changes

The reintroduction of wild-type p53 into the p53-negative osteosarcoma cell line, Saos-2, results in a characteristic enlargement and flattening of these normally spindle-shaped cells (Chen *et al.*, 1990). Subconfluent Saos-2 cells (1×10^5 cells/10-cm plate) were infected at an moi of 50 with either the A/C/53 or control A/M virus, and incubated at 37°C for 72 hr until uninfected control plates were confluent. At this point, the expected morphological change was evident in the A/C/53-treated plate (Fig. 3C), but not in uninfected (Fig. 3A) or control virus-infected plates (Fig. 3B). This effect was not a function of cell density because a control plate initially seeded at lower density retained normal morphology at 72 hr when its confluence approximated that of the A/C/53-treated plate (data not shown). Our previous results had demonstrated a high level of p53 protein expression at a moi of 50 in Saos-2 cells (Fig. 2A), and these results provided evidence that the p53 protein expressed by these recombinant adenoviruses was biologically active.

p53 inhibition of cellular DNA synthesis

To test further the activity of the p53 recombinant adenoviruses, we assayed their ability to inhibit proliferation of human tumor cells as measured by the uptake of [^{3}H]thymidine. It has previously been shown that introduction of wild-type p53 into cells that do not express endogenous wild-type p53 can arrest the cells at the G_1/S transition, leading to inhibition of uptake of labeled thymidine into newly synthesized DNA (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990). We infected a variety of p53-deficient tumor cell lines with either A/M/N/53, A/C/N/53 or a non-p53-expressing control recombinant adenovirus (A/M). We observed a strong, dose-dependent inhibition of DNA synthesis by both the A/M/N/53 and A/C/N/53 recombinants in 7 out of the 9 different tumor cell lines tested (Fig. 4). Both constructs were able to inhibit DNA synthesis specifically in these human tumor cells, regardless of whether they ex-

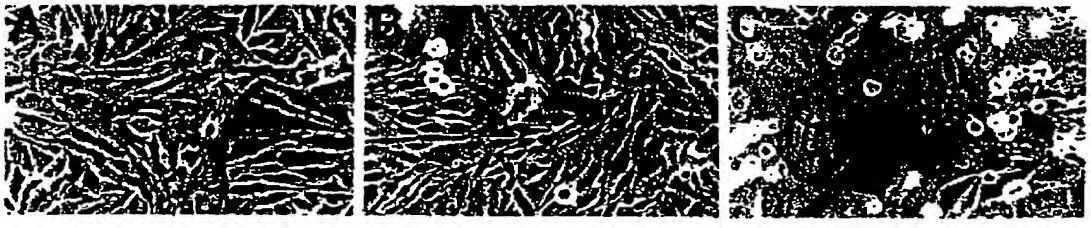

FIG. 3. p53-dependent Saos-2 morphology change. Subconfluent (1×10^5 cells/10-cm plate) Saos-2 cells were either uninfected (A), infected at a moi = 50 with the control A/M virus (B), or the A/C/53 virus (C). The cells were photographed 72 hr post-infection.

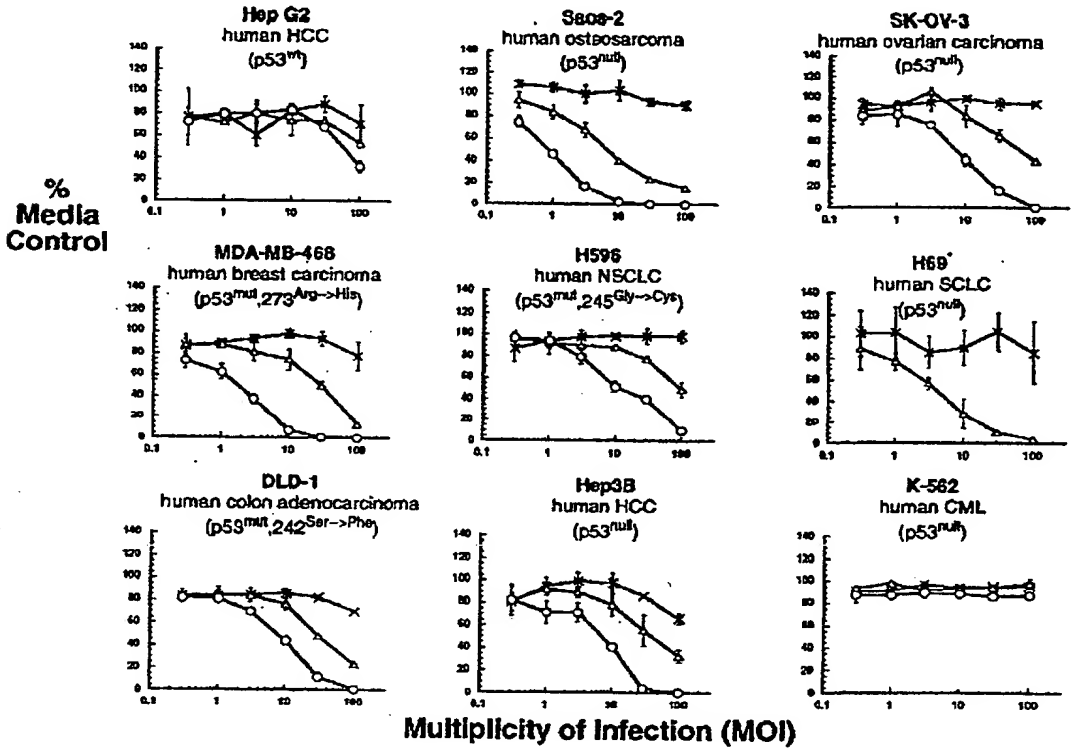

FIG. 4. p53-dependent inhibition of DNA synthesis in human tumor cell lines by A/M/N/53 and A/C/N/53. Nine different tumor cell lines were infected with either control adenovirus A/M/ (xx), or the p53-expressing A/M/N/53 (Δ) or A/C/N/53 (O) virus at increasing moi as indicated. Tumor type and p53 status are noted for each cell line (wt, wild type; null, no protein expressed; mut, mutant protein expressed). DNA synthesis was measured 72 hr post-infection as described in Materials and Methods. Results are from triplicate measurements at each dose (mean ± SD), and are plotted as % of media control *versus* moi. (*) H69 cells were only tested with A/M and A/M/N/53 virus.

pressed mutant p53 or failed to express p53 protein. We also found that in this assay, the A/C/N/53 construct was consistently more potent than the A/M/N/53. In Saos-2 (osteosarcoma) and MDA-MB468 (breast cancer) cells, nearly 100% inhibition of DNA synthesis was achieved with the A/C/N/53 construct at a moi as low as 10. At doses where inhibition by the control adenovirus is only 10–30%, we observed a 50–100% reduction in DNA synthesis using either p53 recombinant adenovirus. In contrast, we observed no significant p53-specific effect with either construct as compared to control virus in HBP G2 cells (hepatocarcinoma cell line expressing endogenous wild-type p53; Bressac *et al.*, 1990), nor in the K562 (p53 null; Feinstein *et al.*, 1992) leukemic cell line.

Tumorigenicity in nude mice

In a more stringent test of function for our p53 recombinant adenoviruses, we infected tumor cells *ex vivo* and then injected the cells into nude mice to assess the ability of the recombinants to suppress tumor growth *in vivo*. Saos-2 cells infected with A/M/N/53 or control A/M virus at a moi of 3 or 30 were injected into opposite flanks of nude mice. Tumor sizes were then measured twice a week over an 8-week period. At a moi of 30, we did not observe any tumor growth in the p53-treated flanks in any of the animals, while the control treated tumors continued to grow (Fig. 5). The progressive enlargement of the control virus-treated tumors was similar to that observed in the buffer-treated control animals. We also observed a clear difference in tumor growth between the control adenovirus and the p53 recombinant at a moi of 3, although tumors from 2 out of the 4 p53-treated mice did start to show some growth after approximately 6 weeks (data not shown). Thus, the A/M/N/53 recombinant adenovirus is able to mediate p53-specific tumor suppression in an *in vivo* environment. We have also observed very similar results when infecting and injecting the NSCLC cell line H596, which expresses mutant p53 protein with the same viruses (unpublished observations).

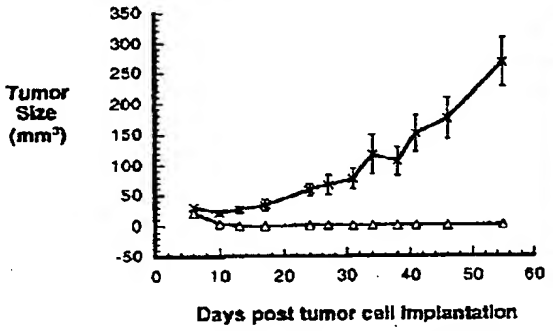

FIG. 5. Tumorigenicity of p53-infected Saos-2 cells in nude mice. Saos-2 cells were infected with either the control A/M virus or the p53 recombinant A/M/N/53 at moi = 30. Treated cells were injected subcutaneously into the flanks of nude mice, and tumor dimensions were measured (as described in Materials and Methods) twice per week for 8 weeks. Results are plotted as tumor size *versus* days post tumor cell implantation for both control A/M-(x) and A/M/N/53-(Δ) treated cells. Error bars represent the mean tumor size ± SEM for each group of 4 animals at each time point.

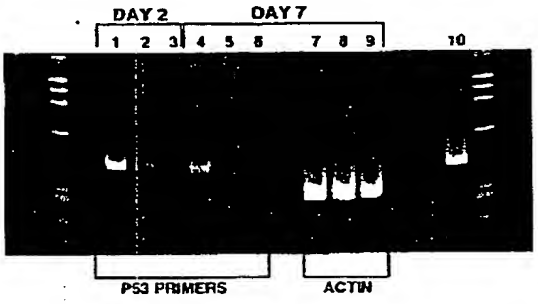

FIG. 6. Expression of rAd/p53 RNA in established tumors. H69 (SCLC) cells were injected subcutaneously into nude mice and allowed to develop tumors for 32 days until reaching a size of approximately 25–50 mm³. Mice were randomized and injected peritumorally with 2×10^9 pfu of either control A/C/β-Gal or A/C/53 virus. Tumors were excised 2 and 7 days post injection, and poly(A) RNA was prepared from each tumor sample. RT-PCR was carried out using equal RNA concentrations and primers specific for recombinant p53 message. PCR amplification was for 30 cycles at 94°C 1 min, 55°C 1.5 min, 72°C 2 min, and a 10-min, 72°C final extension period in an Omnigen thermalcycler (Hybaid). The PCR primers used were a 5′ Tripartite Leader cDNA (5′-CGCCACCGAGGGACCT-GAGCGAGTC-3′) and a 3′ p53 primer (5′-TTCTGGGAAGG-GACAGAAGA-3′). Lanes 1, 2, 4, and 5, p53-treated samples excised at days 2 or 7 as indicated; lanes 3 and 6, from β-Gal-treated tumors; lanes 7, 8, and 9, replicates of lanes 4, 5, and 6, respectively, amplified with actin primers to verify equal loading; lane 10, a positive control using a tripartite/p53 containing plasmid.

In vivo *expression of rAd/p53*

Although *ex vivo* treatment of cancer cells and subsequent injection into animals provided a critical test of tumor suppression, a more clinically relevant experiment is to determine if injected p53 recombinant adenovirus could infect and express p53 in established tumors *in vivo*. To address this, H69 (SCLC, $p53^{null}$) cells were injected subcutaneously into nude mice, and tumors were allowed to develop for 32 days. At this time, a single injection of 2×10^9 pfu of either A/C/53 or A/C/β-Gal adenovirus was injected into the peritumoral space surrounding the tumor. Tumors were then excised at either day 2 or day 7 following the adenovirus injection, and poly(A) RNA was isolated from each tumor. RT-PCR, using recombinant-p53 specific primers, was then used to detect p53 mRNA in the p53-treated tumors (Fig. 6, lanes 1, 2, 4, 5). No p53 signal was evident from the tumors excised from the β-Gal-treated animals (Fig. 6, lanes 3 and 6). Amplification with actin primers served as a control for the RT-PCR reaction (Fig. 6, lanes 7–9), while a plasmid containing the recombinant-p53 sequence served as a positive control for the recombinant-p53-specific band (Fig. 6, lane 10). This experiment demonstrates that a p53 recombinant adenovirus can specifically direct expression of p53 mRNA within established tumors following a single injection into the peritumoral space. It also provides evidence for *in vivo* viral persistence for at least 1 week following infection with a p53 recombinant adenovirus.

In vivo efficacy

To address the feasibility of gene therapy of established tumors, a tumor-bearing nude mouse model was used. H69 cells were injected into the subcutaneous space on the right flank of mice, and tumors were allowed to grow for 2 weeks. Mice then received peritumoral injections of buffer or recombinant virus twice weekly for a total of 8 doses. In the mice treated with buffer or control A/M virus, tumors continued to grow rapidly throughout the treatment, whereas those treated with the A/M/N/53 virus grew at a greatly reduced rate (Fig. 7A). Although control animals treated with buffer alone had accelerated tumor growth as compared to either virus-treated group, we found no significant differences in body weight among the three groups during the treatment period (data not shown). Tumor ulceration in some animals limited the relevance of tumor size measurements after day 42. However, continued monitoring of the animals to determine survival time demonstrated a survival advantages for the p53-treated animals (Fig. 7B). The last of the control adenovirus-treated animals died on day 83, while buffer alone treated controls had all expired by day 56. In contrast, all 5 animals treated with the A/M/N/53 survived up to day 137 before the first animal in this group died (Fig. 7B). Two animals continue to survive at day 174. Together, our data indicate a p53-specific effect on both tumor growth and survival time in animals with established p53-deficient tumors.

DISCUSSION

Adenovirus vectors expressing p53

We have constructed recombinant human adenovirus vectors that are capable of expressing high levels of wild-type p53

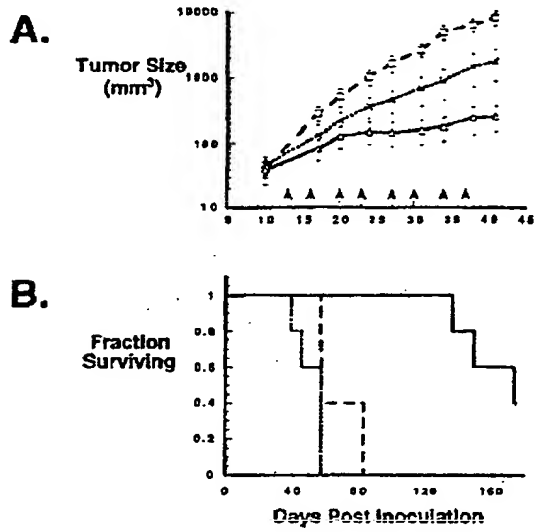

FIG. 7. *In vivo* tumor suppression and increased survival time with A/M/N/53. H69 (SCLC) tumor cells were injected subcutaneously into nude mice and allowed to develop for 2 weeks. Peritumoral injections of either buffer alone (□), control A/M adenovirus (x), or A/M/N/53 (Δ) (both virus 2×10^9 pfu/injection) were administered twice per week for a total of 8 doses. Tumor dimensions were measured twice per week and tumor volume was estimated as described in Materials and Methods. A. Tumor size is plotted for each virus *versus* time (days) post inoculation of H69 cells. Error bars indicate the mean tumor size ± SEM for each group of 5 animals. Arrows indicate days of virus injections. B. Mice were monitored for survival and the fraction of mice surviving per group *versus* time post inoculation of buffer alone (—), control A/M (— —), or A/M/N/53 (——) virus-treated H69 cells is plotted.

protein in a dose-dependent manner. Each vector contains deletions in the E1a and E1b regions that render the virus replication deficient (Challberg and Kelly, 1979; Horowitz, 1991). Of further significance is that these deletions include those sequences encoding the E1b 19- and 55-kD proteins. The 19-kD protein is reported to be involved in inhibiting apoptosis (Rao *et al.*, 1992; White *et al.*, 1992), whereas the 55-kD protein is able to bind wild-type p53 protein (Sarnow *et al.*, 1982; Heuvel *et al.*, 1990). By deleting these adenoviral sequences, we remove potential inhibitors of p53 function through direct binding to p53 or potential inhibition of p53-mediated apoptosis. We have created additional constructs that have had the remaining 3′ E1b sequence, including all protein IX coding sequence, deleted as well. Although this has been reported to reduce the packaging size capacity of adenovirus to approximately 3 kb, less than wild-type virus (Ghosh-Choudhury *et al.*, 1987), these constructs are also deleted in the E3 region so that the A/M/N/53 and A/C/N/53 constructs are well within this size range. By deleting the pIX region, adenoviral sequences homologous to those contained in 293 cells are reduced to approximately 300 bp, decreasing the chances of regenerating replication-competent, wild-type adenovirus through recombination. Constructs lacking pIX coding sequence appear to have equal efficacy and drive equivalent levels of p53 protein expression as those with pIX (unpublished observations).

p53/Adenovirus efficacy in vitro

In concordance with a strong dose dependency for expression of p53 protein in infected cells, we have also demonstrated a dose-dependent, p53-specific inhibition of tumor cell growth by our recombinants. We were able to inhibit cell division, demonstrated by the inhibition of DNA synthesis, in a wide variety of tumor cell types known to lack wild-type p53 protein expression. Bacchetti and Graham (1993) recently reported p53-specific inhibition of DNA synthesis in the ovarian carcinoma cell line SKOV-3 by a p53 recombinant adenovirus in similar experiments. In addition to ovarian carcinoma, we have demonstrated that additional human tumor cell lines, representative of clinically important human cancers and including lines overexpressing mutant p53 protein, can also be growth inhibited by our p53 recombinants. At moi values where the A/C/N/53 recombinant is 90–100% effective in inhibiting DNA synthesis in these tumor types, control adenovirus-mediated suppression is less than 20%.

Although Feinstein *et al.* (1992) reported that reintroduction of wild-type p53 could induce differentiation and increase the proportion of cells in G_1 *versus* $S + G_2$ for leukemic K562 cells, we found no p53-specific effect in this line. Horvath and Weber (1988) have reported that human peripheral blood lymphocytes are highly nonpermissive to adenovirus infection. In separate experiments, we found that we were not able to infect the nonresponding K562 cells significantly with recombinant A/C/β-Gal adenovirus, while other cell lines, including the control Hep G2 line and those showing a strong p53 effect, were readily infectable (Harris *et al.*, in preparation). Thus, at least part of the variability of efficacy would appear to be due to variability of infection, although other factors may be involved as well. For example, Chen *et al.* (1991) reported that wild-type p53 can suppress tumorigenicity without inhibiting the growth rate of some tumor lines. Alternatively, mutations of regulatory proteins acting downstream from p53 may also exist in some tumor cell lines, limiting the effect of p53 treatment. The lack of a p53-specific effect in the wild-type control cell line Hep G2 is encouraging, suggesting that overexpression of wild-type p53 over endogenous background levels may have only minor effects in normal cells infected with the recombinant.

The ability to treat human cancer cells *ex vivo* and suppress their growth *in vivo* when implanted into an animal is an important step toward identifying promising gene therapy candidates. The results observed with the A/M/N/53 virus in Fig. 5 demonstrates that complete suppression is possible in an *in vivo* environment. The resumption of tumor growth in 2 out of the 4 p53-treated animals at the lower moi most likely resulted from a small percentage of cells not initially infected with the p53 recombinant at this dose. We did not analyze the resulting tumors for the presence of adenoviral genomes. The complete suppression seen with A/M/N/53 at the highr dose, however, shows that the ability of tumor growth to recover can be overcome.

p53/Adenovirus in vivo efficacy

Work presented here and by other groups (Chen *et al.*, 1990; Takahashi *et al.*, 1992) have shown that human tumor cells lacking expression of wild-type p53 can be treated *ex vivo* with p53 and result in suppression of tumor growth when the treated cells are transferred into an animal model. This report presents the first evidence of tumor suppressor gene therapy of an *in vivo* established tumor, resulting in both suppression of tumor growth and increased survival time. Delivery to tumor cells did not rely on direct injection into the tumor mass. Rather, p53 recombinant adenovirus was injected into the peritumoral space, and p53 mRNA expression was detected within the tumor. p53 expressed by the recombinants was functional and strongly suppressed tumor growth as compared to that of control, non-p53-expressing adenovirus-treated tumors. However, both p53 and control virus-treated tumor groups showed tumor suppression as compared to buffer-treated controls. It has been demonstrated that local expression of tumor necrosis factor (TNF), interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, or IL-7 can lead to T-cell-independent transient tumor suppression in nude mice (Hoch *et al.*, 1992). Exposure of monocytes to adenovirus results in the release of TNF, and adenovirus virions are also weak inducers of IFN-α/β (for review, see Gooding and Wold, 1990). Therefore, it is not surprising that we observed some tumor suppression in nude mice even with the control adenovirus. We did not observe this virus-mediated tumor suppression in the *ex vivo* control virus-treated Saos-2 tumor cells described earlier. The p53-specific *in vivo* tumor suppression was dramatically demonstrated by continued monitoring of the animals in Fig. 7. The survival time of the p53-treated mice was significantly increased, with 5 out of 5 animals still alive more than 135 days after tumor cell inoculation compared to 0 out of 5 adenovirus control-treated animals. Two out of 5 mice continue to survive beyond day 170, more than twice the survival time of the longest-lived control virus and buffer-treated animals. The surviving animals still exhibit growing tumors, which may reflect cells not initially infected with the p53 recombinant adenovirus. Higher or more frequent dosing schedules may address this. In addition, promoter shutoff (Palmer *et al.*, 1991) or additional mutations may have rendered these cells resistant to the p53 recombinant adenovirus treatment.

Implications for gene therapy

There will be over one million new cases of cancer diagnosed this year, and half that number of cancer-related deaths (American Cancer Society, 1993). p53 mutations are the most common genetic alteration asociated with human cancers, occurring in 50–60% of human cancers (Bartek *et al.*, 1991; Hollstein *et al.*, 1991; Levine, 1993). The goal of gene therapy in treating p53-deficient tumors is to reinstate a normal, functional copy of the wild-type p53 gene so that control of cellular proliferation is restored. p53 plays a central role in cell cycle progression, arresting growth so that repair or apoptosis can occur in response to DNA damage. The possibility of using p53/adenovirus to drive tumor cells into the apoptotic pathway is intriguing. Wild-type p53 has recently been identified as a necessary component for apoptosis induced by irradiation or treatment with some chemotherapeutic agents (Lowe *et al.*, 1993a,b). Due to the high prevalence of p53 mutations in human tumors, it is possible that tumors which have become refractory to chemotherapy and irradiation treatments may have become so due in part to the lack of wild-type p53. By resupplying functional p53 to these tumors, it is possible that they will now become susceptible to apoptosis normally associated with the DNA damage induced by radiation and chemotherapy.

One of the critical points in successful human tumor suppressor gene therapy is the ability to affect a significant fraction of the cancer cells. Toward that goal, recombinant adenoviruses have distinct advantages over other gene delivery methods (for review, see Siegfried, 1993). Adenoviruses have never been shown to induce tumors in humans and have been safely used as live vaccines (Straus, 1984). Replication-deficient recombinant adenoviruses can be produced by replacing the E1 region necessary for replication with the target gene. Adenovirus does not integrate into the human genome as a normal consequence of infection, thereby greatly reducing the risk of insertional mutagenesis possible with retrovirus or AAV vectors. This lack of stable integration also leads to an additional safety feature in that the transferred gene effect will be transient, as the extrachromasomal DNA will be gradually lost with continued division of normal cells. Stable, high-titer recombinant adenovirus can be produced at levels not achievable with retrovirus or AAV, allowing enough material to be produced to treat a large patient population. Others have shown that adenovirus-mediated gene delivery has a strong potential for gene therapy for diseases such as cystic fibrosis (Rosenfeld *et al.*, 1992; Rich *et al.*, 1993) and α_1-antitrypsin deficiency (Lemarchand *et al.*, 1992). Although other alternatives for gene delivery, such as cationic liposome–DNA complexes, are also currently being explored, none as yet appear as effective as adenovirus-mediated gene delivery.

Here, we have shown that recombinant adenoviruses expressing wild-type p53 can efficiently inhibit DNA syntheais and suppress the growth of a broad range of human tumor cell types, including clinically relevant targets. Furthermore, we have shown that the recombinant adenoviruses can express p53 in an *in vivo* established tumor without relying on direct injection into the tumor or prior *ex vivo* treatment of the cancer cells. The p53 expressed is functional and effectively suppressed tumor growth *in vivo* and significantly increased survival time in a nude mouse model of human lung cancer. Although further studies are needed to ensure the safety of this method of gene delivery and address possible problems of immune responses, the data presented here strongly support the concept of adenovirus-mediated p53 gene therapy of p53-deficient tumors in humans.

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